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[Continued on next page]

(54) Title: TUMOR ANTIGENS FOR PREVENTION AND/OR TREATMENT OF CANCER

A. BCZ4 cDNA

ATGGACATTGAAGCATATCTTGAAAGAATTGGCTATAAGAAGTCTAGGAACAAATTGGACTTGGAAACATTAACTGAC
ATTCTTCAACACCAGATCCGAGCTGTTCCCTTTGAGAACCTTAACATCCATTGTGGGGATGCCATGGACTTAGGCTTA
GAGGCCATTTTTGATCAAGTTGTGAGAAGAAATCGGGGTGGATGGTGTCTCCAGGTCAATCATCTTCTGTACTGGGCT
CTGACCACTATTGGTTTTGAGACCACGATGTTGGGAGGGTATGTTTACAGCACTCCAGCCAAAAAATACAGCACTGGC
ATGATTCACCTTCTCCTGCAGGTGACCATTGATGGCAGGAACTACATTGTCGATGCTGGGTTTGGACGCTCATACCAG
ATGTGGCAGCCTCTGGAGTTAATTTCTGGGAAGGATCAGCCTCAGGTGCCTTGTGTCTTCCGTTTGACGGAAGAGAAT
GGATTCTGGTATCTAGACCAAATCAGAAGGGAACAGTACATTCCAAATGAAGAATTTCTTCATTCTGATCTCCTAGAA
GACAGCAAATACCGAAAAATCTACTCCTTTACTCTTAAGCCTCGAACAATTGAAGATTTTGAGTCTATGAATACATAC
CTGCAGACATCTCCATCATCTGTGTTTACTAGTAAATCATTTTGTTCCTTGCAGACCCCAGATGGGGTTCACTGTTTG
GTGGGCTTCACCCTCACCCATAGGAGATTCAATTATAAGGACAATACAGATCTAATAGAGTTCAAGACTCTGAGTGAG
GAAGAAATAGAAAAAGTGCTGAAAAATATATTTAATATTTCCTTGCAGAGAAAGCTTGTGCCCAAACATGGTGATAGA
TTTTTTACTATTTAG

B. BCZ4 Amino Acid Sequence

MDIEAYLERIGYKKSRNKLDLETLTDILQHQIRAVPFENLNIHCGDAMDLGLEAIFDQVVRRNRGGWCLQVNHLLYWA
LTTIGFETTMLGGYVYSTPAKKYSTGMIHLLLQVTIDGRNYIVDAGFGRSYQMWQPLELISGKDQPQVPCVFRLTEEN
GFWYLDQIRREQYIPNEEFLHSDLLEDSKYRKIYSFTLKPRTIEDFESMNTYLQTSPSSVFTSKSFCSLQTPDGVHCL
VGFTLTHRRFNYKDNTDLIEFKTLSEEEIEKVLKNIFNISLQRKLVPKHGDRFFTI

(57) Abstract: The present invention relates to a nucleic acid encoding a polypeptide and the use of the nucleic acid or polypeptide in preventing and/or treating cancer. In particular, the invention relates to improved vectors for the insertion and expression of foreign genes encoding tumor antigens for use in immunotherapeutic treatment of cancer.

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Tumor Antigens for Prevention and / or Treatment of Cancer

RELATED APPLICATIONS

This application claims priority to Ser. Nos. 60/471,119 filed May 16, 2003 and 60/471,193 filed May 16, 2003.

FIELD OF THE INVENTION

The present invention relates to a nucleic acid encoding a polypeptide and the use of the nucleic acid or polypeptide in preventing and / or treating cancer. In particular, the invention relates to improved vectors for the insertion and expression of foreign genes encoding tumor antigens for use in immunotherapeutic treatment of cancer.

BACKGROUND OF THE INVENTION

There has been tremendous increase in last few years in the development of cancer vaccines with Tumour-associated antigens (TAAs) due to the great advances in identification of molecules based on the expression profiling on primary tumours and normal cells with the help of several techniques such as high density microarray, SEREX, immunohistochemistry (IHC), RT-PCR, in-situ hybridization (ISH) and laser capture microscopy (Rosenberg, Immunity, 1999; Sgroi et al, 1999, Schena et al, 1995, Offringa et al, 2000). The TAAs are antigens expressed or over-expressed by tumour cells and could be specific to one or several tumours for example CEA antigen is expressed in colorectal, breast and lung cancers. Sgroi et al (1999) identified several genes differentially expressed in invasive and metastatic carcinoma cells with combined use of laser capture microdissection and cDNA microarrays. Several delivery systems like DNA or viruses could be used for therapeutic vaccination against human cancers (Bonnet et al, 2000) and can elicit immune responses and also break immune tolerance against TAAs. Tumour cells can be rendered more immunogenic by inserting transgenes encoding T cell co-stimulatory molecules such as B7.1 or cytokines such as IFN-γ, IL2, or GM-CSF, among others. Co-expression of a TAA and a cytokine or a co-stimulatory molecule has also been shown to be useful in developing effective therapeutic vaccines (Hodge et al, 95, Bronte et al, 1995, Chamberlain et al, 1996).

There is a need in the art for reagents and methodologies useful in stimulating an immune response to prevent or treat cancers. The present invention provides such reagents and methodologies which overcome many of the difficulties encountered by others in attempting to treat cancer.

SUMMARY OF THE INVENTION

The present invention provides an immunogenic target for administration to a patient to prevent and / or treat cancer. In particular, the immunogenic target is a tumor antigen ("TA") and / or an angiogenesis-associated antigen ("AA"). In one embodiment, the immunogenic target is encoded by **SEQ ID NO.: 29** or **SEQ ID NO.: 31** or has the amino acid sequence of **SEQ ID NO.: 30** or **SEQ ID NO.: 32**. In certain embodiments, the TA and / or AA are administered to a patient as a nucleic acid contained within a plasmid or other delivery vector, such as a recombinant virus. The TA and / or AA may also be administered in combination with an immune stimulator, such as a co-stimulatory molecule or adjuvant.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. A. Nucleotide sequences of AAC2-1 and AAC2-2. **B.** Alignment of predicted amino acid sequence of AAC2-1 and AAC2-2. Missing nucleotides or amino acids are indicated by a "*". Differences between sequences are underlined.

Figure 2. A. Human lymphocytes differentiate into effector cells secreting IFN-γ in response to peptides derived from the AAC2-2 protein. T cells were stimulated with the groups of peptides shown in Table III (groups 1-9). After three rounds of stimulation, the lymphocytes were analyzed for peptide-specific IFN-γ production by ELISPOT. **B.** The graph in the inset shows that activated cells stimulated by peptide Group #6 are capable of antigen-specific CTL activity killing peptide loaded T2 target cells. Peptide EC5 elicits dominant activity in inducing both CTL activity and IFN-γ secretion.

Figure 3. Murine T cells from HLA-A2-Kb transgenic mice recognize and secrete IFN-γ in response to DNA immunization with a human AAC2-2-encoding DNA plasmid. Spleen cells from pEF6-hAAC2-2-immunized mice were re-stimulated with the different groups of peptides. After six days, the cells were harvested and tested for IFN-γ secretion in response to each respective peptide group or a control HLA-A2-binding 9-mer HIV peptide. ELISPOT plates were incubated over- night and developed. Each group responded with high levels of IFN-γ production (over 250 spots) in response to PMA and ionomycin used as a positive control. One of the highly reactive peptides groups (group 6) is also recognized by human lymphocytes from the HLA-A-0201^{+} donors tested so far.

Figure 4. DNA vaccination with a gene encoding human AAC2-2 completely abrogates the growth of implanted B16F10 melanoma cells. This effect is not due to a non-specific immune

response as shown by the inability of plasmid encoding flu-NP protein and the human flk1 (VEGFR-2) to prevent tumor growth.

Figure 5. Survival of mice after implantation of B16F10 melanoma cells into C57BL/6 mice showing the ability of DNA vaccination with a human AAC2-2 vector to completely protect against the effects of tumor growth. This protective effect is antigen-specific and can not be elicited through vaccination with other genes.

Figure 6. T lymphocytes from C57BL/6 mice exhibit effector cell activity and secrete IFN-γ in response to peptides of human AAC2-2 following DNA vaccination with the pEF6-hAAC2-2 expression plasmid. These peptides can exhibit cross-reactivity on B6 MHC class I. The peptides in group 1 and group 5 induce strong reactivity by C57BL/6 T cells.

Figure 7. BFA4 cDNA sequence.

Figure 8. BFA4 amino acid sequence.

Figure 9. Immune response against BFA4 peptides.

Figure 10. BCY1 nucleotide (A) and amino acid (B) sequences.

Figure 11. Immune response against specific BCY1 peptides.

Figure 12. BFA5 cDNA sequence.

Figure 13. BFA5 amino acid sequence.

Figure 14. Immune response against BFA5-derived peptides.

Figure 15. BCZ4 cDNA and amino acid sequence.

Figure 16. Immune response against BCZ4-derived peptides.

Figure 17. BFY3 cDNA and amino acid sequence.

Figure 18. Immune response against BFY3-derived peptides.

DETAILED DESCRIPTION

The present invention provides reagents and methodologies useful for treating and / or preventing cancer. All references cited within this application are incorporated by reference.

In one embodiment, the present invention relates to the induction or enhancement of an immune response against one or more tumor antigens ("TA") to prevent and / or treat cancer. In certain embodiments, one or more TAs may be combined. In preferred embodiments, the immune response results from expression of a TA in a host cell following administration of a nucleic acid vector encoding the tumor antigen or the tumor antigen itself in the form of a peptide or polypeptide, for example.

As used herein, an "antigen" is a molecule such as a polypeptide or a portion thereof that produces an immune response in a host to whom the antigen has been administered. The immune response may include the production of antibodies that bind to at least one epitope of the antigen and / or the generation of a cellular immune response against cells expressing an epitope of the antigen. The response may be an enhancement of a current immune response by, for example, causing increased antibody production, production of antibodies with increased affinity for the antigen, or an increased or more effective cellular response (i.e., increased T cells or T cells with higher anti-tumor activity). An antigen that produces an immune response may alternatively be referred to as being immunogenic or as an immunogen. In describing the present invention, a TA may be referred to as an "immunogenic target".

TA includes both tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs), where a cancerous cell is the source of the antigen. A TAA is an antigen that is expressed on the surface of a tumor cell in higher amounts than is observed on normal cells or an antigen that is expressed on normal cells during fetal development. A TSA is an antigen that is unique to tumor cells and is not expressed on normal cells. TA further includes TAAs or TSAs, antigenic fragments thereof, and modified versions that retain their antigenicity.

TAs are typically classified into five categories according to their expression pattern, function, or genetic origin: cancer-testis (CT) antigens (i.e., MAGE, NY-ESO-1); melanocyte differentiation antigens (i.e., Melan A/MART-1, tyrosinase, gp100); mutational antigens (i.e., MUM-1, p53, CDK-4); overexpressed 'self' antigens (i.e., HER-2/neu, p53); and, viral antigens (i.e., HPV, EBV). For the purposes of practicing the present invention, a suitable TA is any TA that induces or enhances an anti-tumor immune response in a host in whom the TA is expressed. Suitable TAs include, for example, gp100 (Cox et al., *Science*, 264:716-719 (1994)), MART-1/Melan A (Kawakami et al., *J. Exp. Med.*, 180:347-352 (1994)), gp75 (TRP-1) (Wang et al., *J. Exp. Med.*, 186:1131-1140 (1996)), tyrosinase (Wolfel et al., *Eur. J. Immunol.*, 24:759-764 (1994); WO 200175117; WO 200175016; WO 200175007), NY-ESO-1 (WO 98/14464; WO 99/18206), melanoma proteoglycan (Hellstrom et al., *J. Immunol.*, 130:1467-1472 (1983)), MAGE family antigens (i.e., MAGE-1, 2,3,4,6,12, 51; Van der Bruggen et al., *Science*, 254:1643-1647 (1991); U.S. Pat. Nos. 6,235,525; CN 1319611), BAGE family antigens (Boel et al., *Immunity*, 2:167-175 (1995)), GAGE family antigens (i.e., GAGE-1,2; Van den Eynde et al., *J. Exp. Med.*, 182:689-698 (1995); U.S. Pat. No. 6,013,765), RAGE family antigens (i.e., RAGE-1; Gaugler et at., *Immunogenetics*, 44:323-330 (1996); U.S. Pat. No. 5,939,526), N-acetylglucosaminyltransferase-V (Guilloux et at., *J. Exp. Med.*, 183:1173-1183 (1996)), p15

(Robbins et al., *J. Immunol.* 154:5944-5950 (1995)), ß-catenin (Robbins et al., *J. Exp. Med.*, 183:1185-1192 (1996)), MUM-1 (Coulie et al., *Proc. Natl. Acad. Sci.* USA; 92:7976-7980 (1995)), cyclin dependent kinase-4 (CDK4) (Wolfel et al., *Science*, 269:1281-1284 (1995)), p21-*ras* (Fossum et at., *Int. J. Cancer,* 56:40-45 (1994)), BCR-*abl* (Bocchia et al., *Blood*, 85:2680-2684 (1995)), p53 (Theobald et al., *Proc. Natl. Acad. Sci.* USA, 92:11993-11997 (1995)), p185 HER2/neu (erb-B1; Fisk et al., *J. Exp. Med.*, 181:2109-2117 (1995)), epidermal growth factor receptor (EGFR) (Harris et al., Breast Cancer Res. Treat, 29:1-2 (1994)), carcinoembryonic antigens (CEA) (Kwong et al., *J. Natl. Cancer Inst.*, 85:982-990 (1995) U.S. Pat. Nos. 5,756,103; 5,274,087; 5,571,710; 6,071,716; 5,698,530; 6,045,802; EP 263933; EP 346710; and, EP 784483); carcinoma-associated mutated mucins (i.e., MUC-1 gene products; Jerome et al., *J. Immunol.*, 151:1654-1662 (1993)); EBNA gene products of EBV (i.e., EBNA-1; Rickinson et al., *Cancer Surveys*, 13:53-80 (1992)); E7, E6 proteins of human papillomavirus (Ressing et al., *J. Immunol*, 154:5934-5943 (1995)); prostate specific antigen (PSA; Xue et al., *The Prostate*, 30:73-78 (1997)); prostate specific membrane antigen (PSMA; Israeli, et al., *Cancer Res.*, 54:1807-1811 (1994)); idiotypic epitopes or antigens, for example, immunoglobulin idiotypes or T cell receptor idiotypes (Chen et al., *J. Immunol.*, 153:4775-4787 (1994)); KSA (U.S. Patent No. 5,348,887), kinesin 2 (Dietz, et al. Biochem Biophys Res Commun 2000 Sep 7;275(3):731-8), HIP-55, TGFβ-1 anti-apoptotic factor (Toomey, et al. Br J Biomed Sci 2001;58(3):177-83), tumor protein D52 (Bryne J.A., et al., Genomics, 35:523-532 (1996)), H1FT, NY-BR-1 (WO 01/47959), NY-BR-62, NY-BR-75, NY-BR-85, NY-BR-87, NY-BR-96 (Scanlan, M. Serologic and Bioinformatic Approaches to the Identification of Human Tumor Antigens, in *Cancer Vaccines 2000*, Cancer Research Institute, New York, NY), BFA4 (SEQ ID NOS.: 23 and 24), BCY1 (SEQ ID NOS.: 25 and 26), BFA5 (SEQ ID NOS.: 27 and 28), BCZ4 (SEQ ID NOS.: 29 and 30), and BFY3 (SEQ ID NOS. 31 and 32), including "wild-type" (i.e., normally encoded by the genome, naturally-occurring), modified, and mutated versions as well as other fragments and derivatives thereof. Any of these TAs may be utilized alone or in combination with one another in a co-immunization protocol.

In certain cases, it may be beneficial to co-immunize patients with both TA and other antigens, such as angiogenesis-associated antigens ("AA"). An AA is an immunogenic molecule (i.e., peptide, polypeptide) associated with cells involved in the induction and / or continued development of blood vessels. For example, an AA may be expressed on an endothelial cell ("EC"), which is a primary structural component of blood vessels. For treatment of cancer, it is preferred that that the AA be found within or near blood vessels that supply a tumor.

Immunization of a patient against an AA preferably results in an anti-AA immune response whereby angiogenic processes that occur near or within tumors are prevented and / or inhibited.

Exemplary AAs include, for example, vascular endothelial growth factor (i.e., VEGF; Bernardini, et al. *J. Urol.*, 2001, 166(4): 1275-9; Starnes, et al. *J. Thorac. Cardiovasc. Surg.*, 2001, 122(3): 518-23; Dias, et al. *Blood*, 2002, 99: 2179-2184), the VEGF receptor (i.e., VEGF-R, flk-1/KDR; Starnes, et al. *J. Thorac. Cardiovasc. Surg.*, 2001, 122(3): 518-23), EPH receptors (i.e., EPHA2; Gerety, et al. 1999, *Cell*, 4: 403-414), epidermal growth factor receptor (i.e., EGFR; Ciardeillo, et al. Clin. Cancer Res., 2001, 7(10): 2958-70), basic fibroblast growth factor (i.e., bFGF; Davidson, et al. Clin. Exp. Metastasis 2000,18(6): 501-7; Poon, et al. Am J. Surg., 2001, 182(3):298-304), platelet-derived cell growth factor (i.e., PDGF-B), platelet-derived endothelial cell growth factor (PD-ECGF; Hong, et al. J. Mol. Med., 2001, 8(2):141-8), transforming growth factors (i.e., TGF-α; Hong, et al. J. Mol. Med., 2001, 8(2):141-8), endoglin (Balza, et al. *Int. J. Cancer*, 2001, 94: 579-585), Id proteins (Benezra, R. Trends Cardiovasc. Med., 2001, 11(6):237-41), proteases such as uPA, uPAR, and matrix metalloproteinases (MMP-2, MMP-9; Djonov, et al. J. Pathol., 2001, 195(2):147-55), nitric oxide synthase (Am. J. Ophthalmol., 2001, 132(4):551-6), aminopeptidase (Rouslhati, E. Nature Cancer, 2: 84-90, 2002), thrombospondins (i.e., TSP-1, TSP-2; Alvarez, et al. Gynecol. Oncol., 2001, 82(2):273-8; Seki, et al. Int. J. Oncol., 2001, 19(2):305-10), k-*ras* (Zhang, et al. Cancer Res., 2001, 61(16):6050-4), *Wnt* (Zhang, et al. Cancer Res., 2001, 61(16):6050-4), cyclin-dependent kinases (CDKs; Drug Resist. Updat. 2000, 3(2):83-88), microtubules (Timar, et al. 2001. *Path. Oncol. Res.*, 7(2): 85-94), heat shock proteins (i.e., HSP90 (Timar, *supra*)), heparin-binding factors (i.e., heparinase; Gohji, et al. Int. J. Cancer, 2001, 95(5):295-301), synthases (i.e., ATP synthase, thymidilate synthase), collagen receptors, integrins (i.e., αυβ3, αυβ5, α1β1, α2β1, α5β1), the surface proteolglycan NG2, AAC2-1 (SEQ ID NO.:1), or AAC2-2 (SEQ ID NO.:2), among others, including "wild-type" (i.e., normally encoded by the genome, naturally-occurring), modified, mutated versions as well as other fragments and derivatives thereof. Any of these targets may be suitable in practicing the present invention, either alone or in combination with one another or with other agents.

In certain embodiments, a nucleic acid molecule encoding an immunogenic target is utilized. The nucleic acid molecule may comprise or consist of a nucleotide sequence encoding one or more immunogenic targets, or fragments or derivatives thereof, such as that contained in a DNA insert in an ATCC Deposit. The term "nucleic acid sequence" or "nucleic acid molecule" refers to a DNA or RNA sequence. The term encompasses molecules formed from any of the

known base analogs of DNA and RNA such as, but not limited to 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinyl-cytosine, pseudoisocytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxy-methylaminomethyluracil, dihydrouracil, inosine, N6-iso-pentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyamino-methyl-2-thiouracil, beta-D-mannosylqueosine, 5' -methoxycarbonyl-methyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine, among others.

An isolated nucleic acid molecule is one that: (1) is separated from at least about 50 percent of proteins, lipids, carbohydrates, or other materials with which it is naturally found when total nucleic acid is isolated from the source cells; (2) is not linked to all or a portion of a polynucleotide to which the nucleic acid molecule is linked in nature; (3) is operably linked to a polynucleotide which it is not linked to in nature; and / or, (4) does not occur in nature as part of a larger polynucleotide sequence. Preferably, the isolated nucleic acid molecule of the present invention is substantially free from any other contaminating nucleic acid molecule(s) or other contaminants that are found in its natural environment that would interfere with its use in polypeptide production or its therapeutic, diagnostic, prophylactic or research use. As used herein, the term "naturally occurring" or "native" or "naturally found" when used in connection with biological materials such as nucleic acid molecules, polypeptides, host cells, and the like, refers to materials which are found in nature without manipulation by man. Similarly, "non-naturally occurring" or "non-native" as used herein refers to a material that is not found in nature or that has been structurally modified or synthesized by man.

The identity of two or more nucleic acid or polypeptide molecules is determined by comparing the sequences. As known in the art, "identity" means the degree of sequence relatedness between nucleic acid molecules or polypeptides as determined by the match between the units making up the molecules (i.e., nucleotides or amino acid residues). Identity measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., an algorithm). Identity between nucleic acid sequences may also be determined by the ability of the

related sequence to hybridize to the nucleic acid sequence or isolated nucleic acid molecule. In defining such sequences, the term "highly stringent conditions" and "moderately stringent conditions" refer to procedures that permit hybridization of nucleic acid strands whose sequences are complementary, and to exclude hybridization of significantly mismatched nucleic acids. Examples of "highly stringent conditions" for hybridization and washing are 0.015 M sodium chloride, 0.0015 M sodium citrate at 65-68°C or 0.015 M sodium chloride, 0.0015 M sodium citrate, and 50% formamide at 42°C. (see, for example, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual* (2nd ed., Cold Spring Harbor Laboratory, 1989); Anderson *et al.*, *Nucleic Acid Hybridisation: A Practical Approach* Ch. 4 (IRL Press Limited)). The term "moderately stringent conditions" refers to conditions under which a DNA duplex with a greater degree of base pair mismatching than could occur under "highly stringent conditions" is able to form. Exemplary moderately stringent conditions are 0.015 M sodium chloride, 0.0015 M sodium citrate at 50-65°C or 0.015 M sodium chloride, 0.0015 M sodium citrate, and 20% formamide at 37-50°C. By way of example, moderately stringent conditions of 50°C in 0.015 M sodium ion will allow about a 21% mismatch. During hybridization, other agents may be included in the hybridization and washing buffers for the purpose of reducing non-specific and/or background hybridization. Examples are 0.1% bovine serum albumin, 0.1% polyvinyl-pyrrolidone, 0.1% sodium pyrophosphate, 0.1% sodium dodecylsulfate, $NaDodSO_4$, (SDS), ficoll, Denhardt's solution, sonicated salmon sperm DNA (or another non-complementary DNA), and dextran sulfate, although other suitable agents can also be used. The concentration and types of these additives can be changed without substantially affecting the stringency of the hybridization conditions. Hybridization experiments are usually carried out at pH 6.8-7.4; however, at typical ionic strength conditions, the rate of hybridization is nearly independent of pH.

In certain embodiments of the present invention, vectors are used to transfer a nucleic acid sequence encoding a polypeptide to a cell. A vector is any molecule used to transfer a nucleic acid sequence to a host cell. In certain cases, an expression vector is utilized. An expression vector is a nucleic acid molecule that is suitable for transformation of a host cell and contains nucleic acid sequences that direct and / or control the expression of the transferred nucleic acid sequences. Expression includes, but is not limited to, processes such as transcription, translation, and splicing, if introns are present. Expression vectors typically comprise one or more flanking sequences operably linked to a heterologous nucleic acid sequence encoding a polypeptide. Flanking sequences may be homologous (i.e., from the same species and / or strain as the host

cell), heterologous (i.e., from a species other than the host cell species or strain), hybrid (i.e., a combination of flanking sequences from more than one source), or synthetic, for example.

A flanking sequence is preferably capable of effecting the replication, transcription and / or translation of the coding sequence and is operably linked to a coding sequence. As used herein, the term operably linked refers to a linkage of polynucleotide elements in a functional relationship. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. However, a flanking sequence need not necessarily be contiguous with the coding sequence, so long as it functions correctly. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence may still be considered operably linked to the coding sequence. Similarly, an enhancer sequence may be located upstream or downstream from the coding sequence and affect transcription of the sequence.

In certain embodiments, it is preferred that the flanking sequence is a trascriptional regulatory region that drives high-level gene expression in the target cell. The transcriptional regulatory region may comprise, for example, a promoter, enhancer, silencer, repressor element, or combinations thereof. The transcriptional regulatory region may be either constitutive, tissue-specific, cell-type specific (i.e., the region is drives higher levels of transcription in a one type of tissue or cell as compared to another), or regulatable (i.e., responsive to interaction with a compound). The source of a transcriptional regulatory region may be any prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the flanking sequence functions in a cell by causing transcription of a nucleic acid within that cell. A wide variety of transcriptional regulatory regions may be utilized in practicing the present invention.

Suitable transcriptional regulatory regions include, for example, the CMV promoter (i.e., the CMV-immediate early promoter); promoters from eukaryotic genes (i.e., the estrogen-inducible chicken ovalbumin gene, the interferon genes, the gluco-corticoid-inducible tyrosine aminotransferase gene, and the thymidine kinase gene); and the major early and late adenovirus gene promoters; the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-10); the promoter contained in the 3' long terminal repeat (LTR) of Rous sarcoma virus (RSV) (Yamamoto, *et al.*, 1980, *Cell* 22:787-97); the herpes simplex virus thymidine kinase (HSV-TK) promoter (Wagner *et al.*, 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:1444-45); the regulatory sequences of the metallothionine gene (Brinster *et al.*, 1982, *Nature* 296:39-42); prokaryotic expression vectors such as the beta-lactamase promoter (Villa-Kamaroff *et al.*, 1978, *Proc. Natl.*

Acad. Sci. U.S.A., 75:3727-31); or the tac promoter (DeBoer *et al.*, 1983, *Proc. Natl. Acad. Sci. U.S.A.*, 80:21-25). Tissue- and / or cell-type specific transcriptional control regions include, for example, the elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, *Cell* 38:639-46; Ornitz *et al.*, 1986, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409 (1986); MacDonald, 1987, *Hepatology* 7:425-515); the insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, *Nature* 315:115-22); the immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, *Cell* 38:647-58; Adames *et al.*, 1985, *Nature* 318:533-38; Alexander *et al.*, 1987, *Mol. Cell. Biol.*, 7:1436-44); the mouse mammary tumor virus control region in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, *Cell* 45:485-95); the albumin gene control region in liver (Pinkert *et al.*, 1987, *Genes and Devel.* 1:268-76); the alpha-feto-protein gene control region in liver (Krumlauf *et al.*, 1985, *Mol. Cell. Biol.*, 5:1639-48; Hammer *et al.*, 1987, *Science* 235:53-58); the alpha 1-antitrypsin gene control region in liver (Kelsey *et al.*, 1987, *Genes and Devel.* 1:161-71); the beta-globin gene control region in myeloid cells (Mogram *et al.*, 1985, *Nature* 315:338-40; Kollias *et al.*, 1986, *Cell* 46:89-94); the myelin basic protein gene control region in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, *Cell* 48:703-12); the myosin light chain-2 gene control region in skeletal muscle (Sani, 1985, *Nature* 314:283-86); the gonadotropic releasing hormone gene control region in the hypothalamus (Mason *et al.*, 1986, *Science* 234:1372-78), and the tyrosinase promoter in melanoma cells (Hart, I. Semin Oncol 1996 Feb;23(1):154-8; Siders, et al. Cancer Gene Ther 1998 Sep-Oct;5(5):281-91), among others. Inducible promoters that are activated in the presence of a certain compound or condition such as light, heat, radiation, tetracycline, or heat shock proteins, for example, may also be utilized (see, for example, WO 00/10612). Other suitable promoters are known in the art.

As described above, enhancers may also be suitable flanking sequences. Enhancers are cis-acting elements of DNA, usually about 10-300 bp in length, that act on the promoter to increase transcription. Enhancers are typically orientation- and position-independent, having been identified both 5' and 3' to controlled coding sequences. Several enhancer sequences available from mammalian genes are known (i.e., globin, elastase, albumin, alpha-feto-protein and insulin). Similarly, the SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers are useful with eukaryotic promoter sequences. While an enhancer may be spliced into the vector at a position 5' or 3' to nucleic acid coding sequence, it is typically located at a site 5' from the promoter. Other suitable enhancers are known in the art, and would be applicable to the present invention.

While preparing reagents of the present invention, cells may need to be transfected or transformed. Transfection refers to the uptake of foreign or exogenous DNA by a cell, and a cell has been transfected when the exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are well known in the art (i.e., Graham *et al.*, 1973, *Virology* 52:456; Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratories, 1989); Davis *et al.*, *Basic Methods in Molecular Biology* (Elsevier, 1986); and Chu *et al.*, 1981, *Gene* 13:197). Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

In certain embodiments, it is preferred that transfection of a cell results in transformation of that cell. A cell is transformed when there is a change in a characteristic of the cell, being transformed when it has been modified to contain a new nucleic acid. Following transfection, the transfected nucleic acid may recombine with that of the cell by physically integrating into a chromosome of the cell, may be maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. A cell is stably transformed when the nucleic acid is replicated with the division of the cell.

The present invention further provides isolated immunogenic targets in polypeptide form. A polypeptide is considered isolated where it: (1) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is naturally found when isolated from the source cell; (2) is not linked (by covalent or noncovalent interaction) to all or a portion of a polypeptide to which the "isolated polypeptide" is linked in nature; (3) is operably linked (by covalent or noncovalent interaction) to a polypeptide with which it is not linked in nature; or, (4) does not occur in nature. Preferably, the isolated polypeptide is substantially free from any other contaminating polypeptides or other contaminants that are found in its natural environment that would interfere with its therapeutic, diagnostic, prophylactic or research use.

Immunogenic target polypeptides may be mature polypeptides, as defined herein, and may or may not have an amino terminal methionine residue, depending on the method by which they are prepared. Further contemplated are related polypeptides such as, for example, fragments, variants (i.e., allelic, splice), orthologs, homologues, and derivatives, for example, that possess at least one characteristic or activity (i.e., activity, antigenicity) of the immunogenic target. Also related are peptides, which refers to a series of contiguous amino acid residues having a sequence corresponding to at least a portion of the polypeptide from which its sequence is derived. In preferred embodiments, the peptide comprises about 5-10 amino acids, 10-15 amino acids, 15-20

amino acids, 20-30 amino acids, or 30-50 amino acids. In a more preferred embodiment, a peptide comprises 9-12 amino acids, suitable for presentation upon Class I MHC molecules, for example.

A fragment of a nucleic acid or polypeptide comprises a truncation of the sequence (i.e., nucleic acid or polypeptide) at the amino terminus (with or without a leader sequence) and / or the carboxy terminus. Fragments may also include variants (i.e., allelic, splice), orthologs, homologues, and other variants having one or more amino acid additions or substitutions or internal deletions as compared to the parental sequence. In preferred embodiments, truncations and/or deletions comprise about 10 amino acids, 20 amino acids, 30 amino acids, 40 amino acids, 50 amino acids, or more. The polypeptide fragments so produced will comprise about 10 amino acids, 25 amino acids, 30 amino acids, 40 amino acids, 50 amino acids, 60 amino acids, 70 amino acids, or more. Such polypeptide fragments may optionally comprise an amino terminal methionine residue. It will be appreciated that such fragments can be used, for example, to generate antibodies or cellular immune responses to immunogenic target polypeptides.

A variant is a sequence having one or more sequence substitutions, deletions, and/or additions as compared to the subject sequence. Variants may be naturally occurring or artificially constructed. Such variants may be prepared from the corresponding nucleic acid molecules. In preferred embodiments, the variants have from 1 to 3, or from 1 to 5, or from 1 to 10, or from 1 to 15, or from 1 to 20, or from 1 to 25, or from 1 to 30, or from 1 to 40, or from 1 to 50, or more than 50 amino acid substitutions, insertions, additions and/or deletions.

An allelic variant is one of several possible naturally-occurring alternate forms of a gene occupying a given locus on a chromosome of an organism or a population of organisms. A splice variant is a polypeptide generated from one of several RNA transcript resulting from splicing of a primary transcript. An ortholog is a similar nucleic acid or polypeptide sequence from another species. For example, the mouse and human versions of an immunogenic target polypeptide may be considered orthologs of each other. A derivative of a sequence is one that is derived from a parental sequence those sequences having substitutions, additions, deletions, or chemically modified variants. Variants may also include fusion proteins, which refers to the fusion of one or more first sequences (such as a peptide) at the amino or carboxy terminus of at least one other sequence (such as a heterologous peptide).

"Similarity" is a concept related to identity, except that similarity refers to a measure of relatedness which includes both identical matches and conservative substitution matches. If two polypeptide sequences have, for example, 10/20 identical amino acids, and the remainder are all

non-conservative substitutions, then the percent identity and similarity would both be 50%. If in the same example, there are five more positions where there are conservative substitutions, then the percent identity remains 50%, but the percent similarity would be 75% (15/20). Therefore, in cases where there are conservative substitutions, the percent similarity between two polypeptides will be higher than the percent identity between those two polypeptides.

Substitutions may be conservative, or non-conservative, or any combination thereof. Conservative amino acid modifications to the sequence of a polypeptide (and the corresponding modifications to the encoding nucleotides) may produce polypeptides having functional and chemical characteristics similar to those of a parental polypeptide. For example, a "conservative amino acid substitution" may involve a substitution of a native amino acid residue with a non-native residue such that there is little or no effect on the size, polarity, charge, hydrophobicity, or hydrophilicity of the amino acid residue at that position and, in particlar, does not result in decreased immunogenicity. Suitable conservative amino acid substitutions are shown in **Table I**.

Table I

Original Residues	Exemplary Substitutions	Preferred Substitutions
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln	Gln
Asp	Glu	Glu
Cys	Ser, Ala	Ser
Gln	Asn	Asn
Glu	Asp	Asp
Gly	Pro, Ala	Ala
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, 1,4 Diamino-butyric Acid, Gln, Asn	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Leu
Pro	Ala	Gly
Ser	Thr, Ala, Cys	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala, Norleucine	Leu

A skilled artisan will be able to determine suitable variants of polypeptide using well-known techniques. For identifying suitable areas of the molecule that may be changed without destroying biological activity (i.e., MHC binding, immunogenicity), one skilled in the art may target areas not believed to be important for that activity. For example, when similar polypeptides with similar activities from the same species or from other species are known, one skilled in the art may compare the amino acid sequence of a polypeptide to such similar polypeptides. By performing such analyses, one can identify residues and portions of the molecules that are conserved among similar polypeptides. It will be appreciated that changes in areas of the molecule that are not conserved relative to such similar polypeptides would be less likely to adversely affect the biological activity and/or structure of a polypeptide. Similarly, the residues required for binding to MHC are known, and may be modified to improve binding. However, modifications resulting in decreased binding to MHC will not be appropriate in most situations. One skilled in the art would also know that, even in relatively conserved regions, one may substitute chemically similar amino acids for the naturally occurring residues while retaining activity. Therefore, even areas that may be important for biological activity or for structure may

be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

Other preferred polypeptide variants include glycosylation variants wherein the number and/or type of glycosylation sites have been altered compared to the subject amino acid sequence. In one embodiment, polypeptide variants comprise a greater or a lesser number of N-linked glycosylation sites than the subject amino acid sequence. An N-linked glycosylation site is characterized by the sequence Asn-X-Ser or Asn-X-Thr, wherein the amino acid residue designated as X may be any amino acid residue except proline. The substitution of amino acid residues to create this sequence provides a potential new site for the addition of an N-linked carbohydrate chain. Alternatively, substitutions that eliminate this sequence will remove an existing N-linked carbohydrate chain. Also provided is a rearrangement of N-linked carbohydrate chains wherein one or more N-linked glycosylation sites (typically those that are naturally occurring) are eliminated and one or more new N-linked sites are created. To affect O-linked glycosylation of a polypeptide, one would modify serine and / or threonine residues.

Additional preferred variants include cysteine variants, wherein one or more cysteine residues are deleted or substituted with another amino acid (*e.g.*, serine) as compared to the subject amino acid sequence set. Cysteine variants are useful when polypeptides must be refolded into a biologically active conformation such as after the isolation of insoluble inclusion bodies. Cysteine variants generally have fewer cysteine residues than the native protein, and typically have an even number to minimize interactions resulting from unpaired cysteines.

In other embodiments, the isolated polypeptides of the current invention include fusion polypeptide segments that assist in purification of the polypeptides. Fusions can be made either at the amino terminus or at the carboxy terminus of the subject polypeptide variant thereof. Fusions may be direct with no linker or adapter molecule or may be through a linker or adapter molecule. A linker or adapter molecule may be one or more amino acid residues, typically from about 20 to about 50 amino acid residues. A linker or adapter molecule may also be designed with a cleavage site for a DNA restriction endonuclease or for a protease to allow for the separation of the fused moieties. It will be appreciated that once constructed, the fusion polypeptides can be derivatized according to the methods described herein. Suitable fusion segments include, among others, metal binding domains (e.g., a poly-histidine segment), immunoglobulin binding domains (i.e., Protein A, Protein G, T cell, B cell, Fc receptor, or complement protein antibody-binding domains), sugar binding domains (e.g., a maltose binding domain), and/or a "tag" domain (i.e., at least a portion of α-galactosidase, a strep tag peptide, a T7 tag peptide, a FLAG peptide, or other

domains that can be purified using compounds that bind to the domain, such as monoclonal antibodies). This tag is typically fused to the polypeptide upon expression of the polypeptide, and can serve as a means for affinity purification of the sequence of interest polypeptide from the host cell. Affinity purification can be accomplished, for example, by column chromatography using antibodies against the tag as an affinity matrix. Optionally, the tag can subsequently be removed from the purified sequence of interest polypeptide by various means such as using certain peptidases for cleavage. As described below, fusions may also be made between a TA and a co-stimulatory components such as the chemokines CXC10 (IP-10), CCL7 (MCP-3), or CCL5 (RANTES), for example.

A fusion motif may enhance transport of an immunogenic target to an MHC processing compartment, such as the endoplasmic reticulum. These sequences, referred to as tranduction or transcytosis sequences, include sequences derived from HIV tat (see Kim et al. 1997 J. Immunol. 159:1666), *Drosophila* antennapedia (see Schutze-Redelmeier et al. 1996 J. Immunol. 157:650), or human period-1 protein (hPER1; in particular, SRRHHCRSKAKRSRHH).

In addition, the polypeptide or variant thereof may be fused to a homologous polypeptide to form a homodimer or to a heterologous polypeptide to form a heterodimer. Heterologous peptides and polypeptides include, but are not limited to: an epitope to allow for the detection and/or isolation of a fusion polypeptide; a transmembrane receptor protein or a portion thereof, such as an extracellular domain or a transmembrane and intracellular domain; a ligand or a portion thereof which binds to a transmembrane receptor protein; an enzyme or portion thereof which is catalytically active; a polypeptide or peptide which promotes oligomerization, such as a leucine zipper domain; a polypeptide or peptide which increases stability, such as an immunoglobulin constant region; and a polypeptide which has a therapeutic activity different from the polypeptide or variant thereof.

In certain embodiments, it may be advantageous to combine a nucleic acid sequence encoding an immunogenic target, polypeptide, or derivative thereof with one or more co-stimulatory component(s) such as cell surface proteins, cytokines or chemokines in a composition of the present invention. The co-stimulatory component may be included in the composition as a polypeptide or as a nucleic acid encoding the polypeptide, for example. Suitable co-stimulatory molecules include, for instance, polypeptides that bind members of the CD28 family (i.e., CD28, ICOS; Hutloff, et al. *Nature* 1999, 397: 263–265; Peach, et al. *J Exp Med* 1994, 180: 2049–2058) such as the CD28 binding polypeptides B7.1 (CD80; Schwartz, 1992; Chen et al, 1992; Ellis, et al. *J. Immunol.*, 156(8): 2700-9) and B7.2 (CD86; Ellis, et al. *J. Immunol.*, 156(8): 2700-9);

polypeptides which bind members of the integrin family (i.e., LFA-1 (CD11a / CD18); Sedwick, et al. *J Immunol* 1999, 162: 1367–1375; Wülfing, et al. *Science* 1998, 282: 2266–2269; Lub, et al. *Immunol Today* 1995, 16: 479–483) including members of the ICAM family (i.e., ICAM-1, -2 or -3); polypeptides which bind CD2 family members (i.e., CD2, signalling lymphocyte activation molecule (CDw150 or "SLAM"; Aversa, et al. *J Immunol* 1997, 158: 4036–4044)) such as CD58 (LFA-3; CD2 ligand; Davis, et al. *Immunol Today* 1996, 17: 177–187) or SLAM ligands (Sayos, et al. *Nature* 1998, 395: 462–469); polypeptides which bind heat stable antigen (HSA or CD24; Zhou, et al. *Eur J Immunol* 1997, 27: 2524–2528); polypeptides which bind to members of the TNF receptor (TNFR) family (i.e., 4-1BB (CD137; Vinay, et al. *Semin Immunol* 1998, 10: 481–489), OX40 (CD134; Weinberg, et al. *Semin Immunol* 1998, 10: 471–480; Higgins, et al. *J Immunol* 1999, 162: 486–493), and CD27 (Lens, et al. *Semin Immunol* 1998, 10: 491–499)) such as 4-1BBL (4-1BB ligand; Vinay, et al. *Semin Immunol* 1998, 10: 481–48; DeBenedette, et al. *J Immunol* 1997, 158: 551–559), TNFR associated factor-1 (TRAF-1; 4-1BB ligand; Saoulli, et al. *J Exp Med* 1998, 187: 1849–1862, Arch, et al. *Mol Cell Biol* 1998, 18: 558–565), TRAF-2 (4-1BB and OX40 ligand; Saoulli, et al. *J Exp Med* 1998, 187: 1849–1862; Oshima, et al. *Int Immunol* 1998, 10: 517–526, Kawamata, et al. *J Biol Chem* 1998, 273: 5808–5814), TRAF-3 (4-1BB and OX40 ligand; Arch, et al. *Mol Cell Biol* 1998, 18: 558–565; Jang, et al. *Biochem Biophys Res Commun* 1998, 242: 613–620; Kawamata S, et al. *J Biol Chem* 1998, 273: 5808–5814), OX40L (OX40 ligand; Gramaglia, et al. *J Immunol* 1998, 161: 6510–6517), TRAF-5 (OX40 ligand; Arch, et al. *Mol Cell Biol* 1998, 18: 558–565; Kawamata, et al. *J Biol Chem* 1998, 273: 5808–5814), and CD70 (CD27 ligand; Couderc, et al. *Cancer Gene Ther.*, 5(3): 163-75). CD154 (CD40 ligand or "CD40L"; Gurunathan, et al. *J. Immunol.*, 1998, 161: 4563-4571; Sine, et al. *Hum. Gene Ther.*, 2001, 12: 1091-1102) may also be suitable.

One or more cytokines may also be suitable co-stimulatory components or "adjuvants", either as polypeptides or being encoded by nucleic acids contained within the compositions of the present invention (Parmiani, et al. Immunol Lett 2000 Sep 15; 74(1): 41-4; Berzofsky, et al. Nature Immunol. 1: 209-219). Suitable cytokines include, for example, interleukin-2 (IL-2) (Rosenberg, et al. *Nature Med.* 4: 321-327 (1998)), IL-4, IL-7, IL-12 (reviewed by Pardoll, 1992; Harries, et al. J. Gene Med. 2000 Jul-Aug;2(4):243-9; Rao, et al. *J. Immunol.* 156: 3357-3365 (1996)), IL-15 (Xin, et al. *Vaccine*, 17:858-866, 1999), IL-16 (Cruikshank, et al. J. Leuk Biol. 67(6): 757-66, 2000), IL-18 (*J. Cancer Res. Clin. Oncol.* 2001. 127(12): 718-726), GM-CSF (CSF (Disis, et al. *Blood*, 88: 202-210 (1996)), tumor necrosis factor-alpha (TNF-α), or

interferons such as IFN-α or INF-γ. Other cytokines may also be suitable for practicing the present invention, as is known in the art.

Chemokines may also be utilized. For example, fusion proteins comprising CXCL10 (IP-10) and CCL7 (MCP-3) fused to a tumor self-antigen have been shown to induce anti-tumor immunity (Biragyn, et al. *Nature Biotech.* 1999, 17: 253-258). The chemokines CCL3 (MIP-1α) and CCL5 (RANTES) (Boyer, et al. *Vaccine*, 1999, 17 (Supp. 2): S53-S64) may also be of use in practicing the present invention. Other suitable chemokines are known in the art.

It is also known in the art that suppressive or negative regulatory immune mechanisms may be blocked, resulting in enhanced immune responses. For instance, treatment with anti-CTLA-4 (Shrikant, et al. *Immunity*, 1996, 14: 145-155; Sutmuller, et al. *J. Exp. Med.*, 2001, 194: 823-832), anti-CD25 (Sutmuller, *supra*), anti-CD4 (Matsui, et al. *J. Immunol.*, 1999, 163: 184-193), the fusion protein IL13Ra2-Fc (Terabe, et al. *Nature Immunol.*, 2000, 1: 515-520), and combinations thereof (i.e., anti-CTLA-4 and anti-CD25, Sutmuller, *supra*) have been shown to upregulate anti-tumor immune responses and would be suitable in practicing the present invention.

Any of these components may be used alone or in combination with other agents. For instance, it has been shown that a combination of CD80, ICAM-1 and LFA-3 ("TRICOM") may potentiate anti-cancer immune responses (Hodge, et al. *Cancer Res.* 59: 5800-5807 (1999). Other effective combinations include, for example, IL-12 + GM-CSF (Ahlers, et al. *J. Immunol.*, 158: 3947-3958 (1997); Iwasaki, et al. *J. Immunol.* 158: 4591-4601 (1997)), IL-12 + GM-CSF + TNF-α (Ahlers, et al. *Int. Immunol.* 13: 897-908 (2001)), CD80 + IL-12 (Fruend, et al. *Int. J. Cancer*, 85: 508-517 (2000); Rao, et al. *supra*), and CD86 + GM-CSF + IL-12 (Iwasaki, supra). One of skill in the art would be aware of additional combinations useful in carrying out the present invention.In addition, the skilled artisan would be aware of additional reagents or methods that may be used to modulate such mechanisms. These reagents and methods, as well as others known by those of skill in the art, may be utilized in practicing the present invention.

Additional strategies for improving the efficiency of nucleic acid-based immunization may also be used including, for example, the use of self-replicating viral replicons (Caley, et al. 1999. *Vaccine*, 17: 3124-2135; Dubensky, et al. 2000. *Mol. Med.* 6: 723-732; Leitner, et al. 2000. *Cancer Res.* 60: 51-55), codon optimization (Liu, et al. 2000. *Mol. Ther.*, 1: 497-500; Dubensky, *supra*; Huang, et al. 2001. *J. Virol.* 75: 4947-4951), *in vivo* electroporation (Widera, et al. 2000. *J. Immunol.* 164: 4635-3640), incorporation of CpG stimulatory motifs (Gurunathan, et al. *Ann. Rev. Immunol.*, 2000, 18: 927-974; Leitner, *supra*; Cho, et al. J. Immunol.

168(10):4907-13), sequences for targeting of the endocytic or ubiquitin-processing pathways (Thomson, et al. 1998. *J. Virol.* 72: 2246-2252; Velders, et al. 2001. *J. Immunol.* 166: 5366-5373), Marek's disease virus type 1 VP22 sequences (J. Virol. 76(6):2676-82, 2002), prime-boost regimens (Gurunathan, *supra*; Sullivan, et al. 2000. *Nature*, 408: 605-609; Hanke, et al. 1998. *Vaccine*, 16: 439-445; Amara, et al. 2001. *Science*, 292: 69-74), and the use of mucosal delivery vectors such as *Salmonella* (Darji, et al. 1997. *Cell*, 91: 765-775; Woo, et al. 2001. *Vaccine*, 19: 2945-2954). Other methods are known in the art, some of which are described below.

Chemotherapeutic agents, radiation, anti-angiogenic compounds, or other agents may also be utilized in treating and / or preventing cancer using immunogenic targets (Sebti, et al. Oncogene 2000 Dec 27;19(56):6566-73). For example, in treating metastatic breast cancer, useful chemotherapeutic agents include cyclophosphamide, doxorubicin, paclitaxel, docetaxel, navelbine, capecitabine, and mitomycin C, among others. Combination chemotherapeutic regimens have also proven effective including cyclophosphamide + methotrexate + 5-fluorouracil; cyclophosphamide + doxorubicin + 5-fluorouracil; or, cyclophosphamide + doxorubicin, for example. Other compounds such as prednisone, a taxane, navelbine, mitomycin C, or vinblastine have been utlized for various reasons. A majority of breast cancer patients have estrogen-receptor positive (ER+) tumors and in these patients, endocrine therapy (i.e., tamoxifen) is preferred over chemotherapy. For such patients, tamoxifen or, as a second line therapy, progestins (medroxyprogesterone acetate or megestrol acetate) are preferred. Aromatase inhibitors (i.e., aminoglutethimide and analogs thereof such as letrozole) decrease the availability of estrogen needed to maintain tumor growth and may be used as second or third line endocrine therapy in certain patients.

Other cancers may require different chemotherapeutic regimens. For example, metastatic colorectal cancer is typically treated with Camptosar (irinotecan or CPT-11), 5-fluorouracil or leucovorin, alone or in combination with one another. Proteinase and integrin inhibitors such as as the MMP inhibitors marimastate (British Biotech), COL-3 (Collagenex), Neovastat (Aeterna), AG3340 (Agouron), BMS-275291 (Bristol Myers Squibb), CGS 27023A (Novartis) or the integrin inhibitors Vitaxin (Medimmune), or MED1522 (Merck KgaA) may also be suitable for use. As such, immunological targeting of immunogenic targets associated with colorectal cancer could be performed in combination with a treatment using those chemotherapeutic agents. Similarly, chemotherapeutic agents used to treat other types of cancers are well-known in the art and may be combined with the immunogenic targets described herein.

Many anti-angiogenic agents are known in the art and would be suitable for co-administration with the immunogenic target vaccines (see, for example, Timar, et al. 2001. *Pathology Oncol. Res.*, 7(2): 85-94). Such agents include, for example, physiological agents such as growth factors (i.e., ANG-2, NK1,2,4 (HGF), transforming growth factor beta (TGF-β)), cytokines (i.e., interferons such as IFN-α, -β, -γ, platelet factor 4 (PF-4), PR-39), proteases (i.e., cleaved AT-III, collagen XVIII fragment (Endostatin)), HmwKallikrein-d5 plasmin fragment (Angiostatin), prothrombin-F1-2, TSP-1), protease inhibitors (i.e., tissue inhibitor of metalloproteases such as TIMP-1, -2, or –3; maspin; plasminogen activator-inhibitors such as PAI-1; pigment epithelium derived factor (PEDF)), Tumstatin (available through ILEX, Inc.), antibody products (i.e., the collagen-binding antibodies HUIV26, HUI77, XL313; anti-VEGF; anti-integrin (i.e., Vitaxin, (Lxsys))), and glycosidases (i.e., heparinase-I, -III). "Chemical" or modified physiological agents known or believed to have anti-angiogenic potential include, for example, vinblastine, taxol, ketoconazole, thalidomide, dolestatin, combrestatin A, rapamycin (Guba, et al. 2002, *Nature Med.*, 8: 128-135), CEP-7055 (available from Cephalon, Inc.), flavone acetic acid, Bay 12-9566 (Bayer Corp.), AG3340 (Agouron, Inc.), CGS 27023A (Novartis), tetracylcine derivatives (i.e., COL-3 (Collagenix, Inc.)), Neovastat (Aeterna), BMS-275291 (Bristol-Myers Squibb), low dose 5-FU, low dose methotrexate (MTX), irsofladine, radicicol, cyclosporine, captopril, celecoxib, D45152-sulphated polysaccharide, cationic protein (Protamine), cationic peptide-VEGF, Suramin (polysulphonated napthyl urea), compounds that interfere with the function or production of VEGF (i.e., SU5416 or SU6668 (Sugen), PTK787/ZK22584 (Novartis)), Distamycin A, Angiozyme (ribozyme), isoflavinoids, staurosporine derivatives, genistein, EMD121974 (Merck KcgaA), tyrphostins, isoquinolones, retinoic acid, carboxyamidotriazole, TNP-470, octreotide, 2-methoxyestradiol, aminosterols (i.e., squalamine), glutathione analogues (i.e., N-acteyl-L-cysteine), combretastatin A-4 (Oxigene), Eph receptor blocking agents (*Nature*, 414:933-938, 2001), Rh-Angiostatin, Rh-Endostatin (WO 01/93897), cyclic-RGD peptide, accutin-disintegrin, benzodiazepenes, humanized anti-avb3 Ab, Rh-PAI-2, amiloride, p-amidobenzamidine, anti-uPA ab, anti-uPAR Ab, L-phanylalanin-N-methylamides (i.e., Batimistat, Marimastat), AG3340, and minocycline. Many other suitable agents are known in the art and would suffice in practicing the present invention.

The present invention may also be utilized in combination with "non-traditional" methods of treating cancer. For example, it has recently been demonstrated that administration of certain anaerobic bacteria may assist in slowing tumor growth. In one study, *Clostridium novyi* was modified to eliminate a toxin gene carried on a phage episome and administered to mice with

colorectal tumors (Dang, et al. *P.N.A.S. USA*, 98(26): 15155-15160, 2001). In combination with chemotherapy, the treatment was shown to cause tumor necrosis in the animals. The reagents and methodologies described in this application may be combined with such treatment methodologies.

Nucleic acids encoding immunogenic targets may be administered to patients by any of several available techniques. Various viral vectors that have been successfully utilized for introducing a nucleic acid to a host include retrovirus, adenovirus, adeno-associated virus (AAV), herpes virus, and poxvirus, among others. It is understood in the art that many such viral vectors are available in the art. The vectors of the present invention may be constructed using standard recombinant techniques widely available to one skilled in the art. Such techniques may be found in common molecular biology references such as *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), and *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA).

Preferred retroviral vectors are derivatives of lentivirus as well as derivatives of murine or avian retroviruses. Examples of suitable retroviral vectors include, for example, Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), SIV, BIV, HIV and Rous Sarcoma Virus (RSV). A number of retroviral vectors can incorporate multiple exogenous nucleic acid sequences. As recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided by, for example, helper cell lines encoding retrovirus structural genes. Suitable helper cell lines include Ψ2, PA317 and PA12, among others. The vector virions produced using such cell lines may then be used to infect a tissue cell line, such as NIH 3T3 cells, to produce large quantities of chimeric retroviral virions. Retroviral vectors may be administered by traditional methods (i.e., injection) or by implantation of a "producer cell line" in proximity to the target cell population (Culver, K., *et al.*, 1994, *Hum. Gene Ther.*, 5 (3): 343-79; Culver, K., *et al.*, *Cold Spring Harb. Symp. Quant. Biol.*, 59: 685-90); Oldfield, E., 1993, *Hum. Gene Ther.*, 4 (1): 39-69). The producer cell line is engineered to produce a viral vector and releases viral particles in the vicinity of the target cell. A portion of the released viral particles contact the target cells and infect those cells, thus delivering a nucleic acid of the present invention to the target cell. Following infection of the target cell, expression of the nucleic acid of the vector occurs.

Adenoviral vectors have proven especially useful for gene transfer into eukaryotic cells (Rosenfeld, M., *et al.*, 1991, *Science*, 252 (5004): 431-4; Crystal, R., *et al.*, 1994, *Nat. Genet.*, 8

(1): 42-51), the study eukaryotic gene expression (Levrero, M., *et al.*, 1991, *Gene*, 101 (2): 195-202), vaccine development (Graham, F. and Prevec, L., 1992, *Biotechnology*, 20: 363-90), and in animal models (Stratford-Perricaudet, L., *et al.,* 1992, *Bone Marrow Transplant.,* 9 (Suppl. 1): 151-2 ; Rich, D., *et al.*, 1993, *Hum. Gene Ther.,* 4 (4): 461-76). Experimental routes for administrating recombinant Ad to different tissues *in vivo* have included intratracheal instillation (Rosenfeld, M., *et al.,* 1992, *Cell*, 68 (1): 143-55) injection into muscle (Quantin, B., *et al.*, 1992, *Proc. Natl. Acad. Sci. U.S.A.,* 89 (7): 2581-4), peripheral intravenous injection (Herz, J., and Gerard, R., 1993, *Proc. Natl. Acad. Sci. U.S.A.,* 90 (7): 2812-6) and stereotactic inoculation to brain (Le Gal La Salle, G., *et al.*, 1993, *Science,* 259 (5097): 988-90), among others.

Adeno-associated virus (AAV) demonstrates high-level infectivity, broad host range and specificity in integrating into the host cell genome (Hermonat, P., et al., 1984, *Proc. Natl. Acad. Sci. U.S.A.,* 81 (20): 6466-70). And Herpes Simplex Virus type-1 (HSV-1) is yet another attractive vector system, especially for use in the nervous system because of its neurotropic property (Geller, A., *et al.,* 1991, *Trends Neurosci.,* 14 (10): 428-32; Glorioso, *et al.*, 1995, *Mol. Biotechnol.,* 4 (1): 87-99; Glorioso, *et al.*, 1995, *Annu. Rev. Microbiol.,* 49: 675-710).

Poxvirus is another useful expression vector (Smith, et al. 1983, *Gene,* 25 (1): 21-8; Moss, et al, 1992, *Biotechnology,* 20: 345-62; Moss, et al, 1992, *Curr. Top. Microbiol. Immunol.*, 158: 25-38; Moss, et al. 1991. *Science*, 252: 1662-1667). Poxviruses shown to be useful include vaccinia, NYVAC, avipox, fowlpox, canarypox, ALVAC, and ALVAC(2), among others.

NYVAC (vP866) was derived from the Copenhagen vaccine strain of vaccinia virus by deleting six nonessential regions of the genome encoding known or potential virulence factors (see, for example, U.S. Pat. Nos. 5,364,773 and 5,494,807). The deletion loci were also engineered as recipient loci for the insertion of foreign genes. The deleted regions are: thymidine kinase gene (TK; J2R); hemorrhagic region (u; B13R+B14R); A type inclusion body region (ATI; A26L); hemagglutinin gene (HA; A56R); host range gene region (C7L-K1L); and, large subunit, ribonucleotide reductase (I4L). NYVAC is a genetically engineered vaccinia virus strain that was generated by the specific deletion of eighteen open reading frames encoding gene products associated with virulence and host range. NYVAC has been show to be useful for expressing TAs (see, for example, U.S. Pat. No. 6,265,189). NYVAC (vP866), vP994, vCP205, vCP1433, placZH6H4Lreverse, pMPC6H6K3E3 and pC3H6FHVB were also deposited with the ATCC under the terms of the Budapest Treaty, accession numbers VR-2559, VR-2558, VR-2557, VR-2556, ATCC-97913, ATCC-97912, and ATCC-97914, respectively.

ALVAC-based recombinant viruses (i.e., ALVAC-1 and ALVAC-2) are also suitable for use in practicing the present invention (see, for example, U.S. Pat. No. 5,756,103). ALVAC(2) is identical to ALVAC(1) except that ALVAC(2) genome comprises the vaccinia E3L and K3L genes under the control of vaccinia promoters (U.S. Pat. No. 6,130,066; Beattie et al., 1995a, 1995b, 1991; Chang et al., 1992; Davies et al., 1993). Both ALVAC(1) and ALVAC(2) have been demonstrated to be useful in expressing foreign DNA sequences, such as TAs (Tartaglia et al., 1993 a,b; U.S. Pat. No. 5,833,975). ALVAC was deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110-2209, USA, ATCC accession number VR-2547.

Another useful poxvirus vector is TROVAC. TROVAC refers to an attenuated fowlpox that was a plaque-cloned isolate derived from the FP-1 vaccine strain of fowlpoxvirus which is licensed for vaccination of 1 day old chicks. TROVAC was likewise deposited under the terms of the Budapest Treaty with the ATCC, accession number 2553.

"Non-viral" plasmid vectors may also be suitable in practicing the present invention. Preferred plasmid vectors are compatible with bacterial, insect, and / or mammalian host cells. Such vectors include, for example, PCR-II, pCR3, and pcDNA3.1 (Invitrogen, San Diego, CA), pBSII (Stratagene, La Jolla, CA), pET15 (Novagen, Madison, WI), pGEX (Pharmacia Biotech, Piscataway, NJ), pEGFP-N2 (Clontech, Palo Alto, CA), pETL (BlueBacII, Invitrogen), pDSR-alpha (PCT pub. No. WO 90/14363) and pFastBacDual (Gibco-BRL, Grand Island, NY) as well as Bluescript® plasmid derivatives (a high copy number COLE1-based phagemid, Stratagene Cloning Systems, La Jolla, CA), PCR cloning plasmids designed for cloning Taq-amplified PCR products (*e.g.*, TOPO™ TA cloning® kit, PCR2.1® plasmid derivatives, Invitrogen, Carlsbad, CA). Bacterial vectors may also be used with the current invention. These vectors include, for example, *Shigella, Salmonella, Vibrio cholerae, Lactobacillus, Bacille calmette guérin (BCG), and Streptococcus* (see for example, WO 88/6626; WO 90/0594; WO 91/13157; WO 92/1796; and WO 92/21376). Many other non-viral plasmid expression vectors and systems are known in the art and could be used with the current invention.

Suitable nucleic acid delivery techniques include DNA-ligand complexes, adenovirus-ligand-DNA complexes, direct injection of DNA, $CaPO_4$ precipitation, gene gun techniques, electroporation, and colloidal dispersion systems, among others. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome, which are artificial membrane vesicles useful as

delivery vehicles *in vitro* and *in vivo*. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, R., *et al.,* 1981, *Trends Biochem. Sci.*, 6: 77). The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations. Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

An immunogenic target may also be administered in combination with one or more adjuvants to boost the immune response. Exemplary adjuvants are shown in Table II below:

Table II

Types of Immunologic Adjuvants

Type of Adjuvant	General Examples	Specific Examples/References
Gel-type	Aluminum hydroxide/phosphate ("alum adjuvants")	(Aggerbeck and Heron, 1995)
	Calcium phosphate	(Relyveld, 1986)
Microbial	Muramyl dipeptide (MDP)	(Chedid et al., 1986)
	Bacterial exotoxins	Cholera toxin (CT), *E.coli* labile toxin (LT)(Freytag and Clements, 1999)
	Endotoxin-based adjuvants	Monophosphoryl lipid A (MPL) (Ulrich and Myers, 1995)
	Other bacterial	CpG oligonucleotides (Corral and Petray, 2000), BCG sequences (Krieg, et al. *Nature*, 374:576), tetanus toxoid (Rice, et al. *J. Immunol.* , 2001, 167: 1558-1565)
Particulate	Biodegradable Polymer microspheres	(Gupta et al., 1998)
	Immunostimulatory complexes (ISCOMs)	(Morein and Bengtsson, 1999)
	Liposomes	(Wassef et al., 1994)
Oil-emulsion and surfactant-based adjuvants	Freund's incomplete adjuvant	(Jensen et al., 1998)
	Microfluidized emulsions	MF59 (Ott et al., 1995)
		SAF (Allison and Byars, 1992) (Allison, 1999)
	Saponins	QS-21 (Kensil, 1996)
Synthetic	Muramyl peptide derivatives	Murabutide (Lederer, 1986) Threony-MDP (Allison, 1997)
	Nonionic block copolymers	L121 (Allison, 1999)
	Polyphosphazene (PCPP)	(Payne et al., 1995)
	Synthetic polynucleotides	Poly A:U, Poly I:C (Johnson, 1994)
	Thalidomide derivatives	CC-4047/ACTIMID (J. Immunol., 168(10):4914-9)

The immunogenic targets of the present invention may also be used to generate antibodies for use in screening assays or for immunotherapy. Other uses would be apparent to one of skill in the art. The term "antibody" includes antibody fragments, as are known in the art, including Fab, Fab_2, single chain antibodies (Fv for example), humanized antibodies, chimeric antibodies, human antibodies, produced by several methods as are known in the art. Methods of preparing and utilizing various types of antibodies are well-known to those of skill in the art and would be suitable in practicing the present invention (see, for example, Harlow, et al. *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988; Harlow, et al. *Using Antibodies: A Laboratory Manual, Portable Protocol No. 1*, 1998; Kohler and Milstein, Nature, 256:495 (1975)); Jones et al. Nature, 321:522-525 (1986); Riechmann et al. Nature, 332:323-329 (1988); Presta (Curr. Op. Struct. Biol., 2:593-596 (1992); Verhoeyen et al. (Science, 239:1534-

1536 (1988); Hoogenboom et al., J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991); Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boerner et al., J. Immunol., 147(1):86-95 (1991); Marks et al., Bio/Technology 10, 779-783 (1992); Lonberg et al., Nature 368 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild et al., Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14, 826 (1996); Lonberg and Huszar, Intern. Rev. Immunol. 13 65-93 (1995); as well as U.S. Pat. Nos. 4,816,567; 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and, 5,661,016). The antibodies or derivatives therefrom may also be conjugated to therapeutic moieties such as cytotoxic drugs or toxins, or active fragments thereof such as diptheria A chain, exotoxin A chain, ricin A chain, abrin A chain, curcin, crotin, phenomycin, enomycin, among others. Cytotoxic agents may also include radiochemicals. Antibodies and their derivatives may be incorporated into compositions of the invention for use *in vitro* or *in vivo*.

Nucleic acids, proteins, or derivatives thereof representing an immunogenic target may be used in assays to determine the presence of a disease state in a patient, to predict prognosis, or to determine the effectiveness of a chemotherapeutic or other treatment regimen. Expression profiles, performed as is known in the art, may be used to determine the relative level of expression of the immunogenic target. The level of expression may then be correlated with base levels to determine whether a particular disease is present within the patient, the patient's prognosis, or whether a particular treatment regimen is effective. For example, if the patient is being treated with a particular chemotherapeutic regimen, a decreased level of expression of an immunogenic target in the patient's tissues (i.e., in peripheral blood) may indicate the regimen is decreasing the cancer load in that host. Similarly, if the level of expresssion is increasing, another therapeutic modality may need to be utilized. In one embodiment, nucleic acid probes corresponding to a nucleic acid encoding an immunogenic target may be attached to a biochip, as is known in the art, for the detection and quantification of expression in the host.

It is also possible to use nucleic acids, proteins, derivatives therefrom, or antibodies thereto as reagents in drug screening assays. The reagents may be used to ascertain the effect of a drug candidate on the expression of the immunogenic target in a cell line, or a cell or tissue of a patient. The expression profiling technique may be combined with high throughput screening techniques to allow rapid identification of useful compounds and monitor the effectiveness of treatment with a drug candidate (see, for example, Zlokarnik, et al., Science 279, 84-8 (1998)). Drug candidates may be chemical compounds, nucleic acids, proteins, antibodies, or derivatives therefrom, whether naturally occurring or synthetically derived. Drug candidates thus identified

may be utilized, among other uses, as pharmaceutical compositions for administration to patients or for use in further screening assays.

Administration of a composition of the present invention to a host may be accomplished using any of a variety of techniques known to those of skill in the art. The composition(s) may be processed in accordance with conventional methods of pharmacy to produce medicinal agents for administration to patients, including humans and other mammals (i.e., a "pharmaceutical composition"). The pharmaceutical composition is preferably made in the form of a dosage unit containing a given amount of DNA, viral vector particles, polypeptide or peptide, for example. A suitable daily dose for a human or other mammal may vary widely depending on the condition of the patient and other factors, but, once again, can be determined using routine methods.

The pharmaceutical composition may be administered orally, parentally, by inhalation spray, rectally, intranodally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term "pharmaceutically acceptable carrier" or "physiologically acceptable carrier" as used herein refers to one or more formulation materials suitable for accomplishing or enhancing the delivery of a nucleic acid, polypeptide, or peptide as a pharmaceutical composition. A "pharmaceutical composition" is a composition comprising a therapeutically effective amount of a nucleic acid or polypeptide. The terms "effective amount" and "therapeutically effective amount" each refer to the amount of a nucleic acid or polypeptide used to induce or enhance an effective immune response. It is preferred that compositions of the present invention provide for the induction or enhancement of an anti-tumor immune response in a host which protects the host from the development of a tumor and / or allows the host to eliminate an existing tumor from the body.

For oral administration, the pharmaceutical composition may be of any of several forms including, for example, a capsule, a tablet, a suspension, or liquid, among others. Liquids may be administered by injection as a composition with suitable carriers including saline, dextrose, or water. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, intrasternal, infusion, or intraperitoneal administration. Suppositories for rectal administration of the drug can be prepared by mixing the drug with a suitable non-irritating excipient such as cocoa butter and polyethylene glycols that are solid at ordinary temperatures but liquid at the rectal temperature.

The dosage regimen for immunizing a host or otherwise treating a disorder or a disease with a composition of this invention is based on a variety of factors, including the type of disease, the age, weight, sex, medical condition of the patient, the severity of the condition, the route of

administration, and the particular compound employed. For example, a poxviral vector may be administered as a composition comprising 1 x 10^6 infectious particles per dose. Thus, the dosage regimen may vary widely, but can be determined routinely using standard methods.

A prime-boost regimen may also be utilized (see, for example, WO 01/30382 A1) in which the targeted immunogen is initially administered in a priming step in one form followed by a boosting step in which the targeted immunogen is administered in another form. The form of the targeted immunogen in the priming and boosting steps are different. For instance, if the priming step utilized a nucleic acid, the boost may be administered as a peptide. Similarly, where a priming step utilized one type of recombinant virus (i.e., ALVAC), the boost step may utilize another type of virus (i.e., NYVAC). This prime-boost method of administration has been shown to induce strong immunological responses.

While the compositions of the invention can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more other compositions or agents (i.e., other immunogenic targets, co-stimulatory molecules, adjuvants). When administered as a combination, the individual components can be formulated as separate compositions administered at the same time or different times, or the components can be combined as a single composition.

Injectable preparations, such as sterile injectable aqueous or oleaginous suspensions, may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent. Suitable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution, among others. For instance, a viral vector such as a poxvirus may be prepared in 0.4% NaCl. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed, including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

For topical administration, a suitable topical dose of a composition may be administered one to four, and preferably two or three times daily. The dose may also be administered with intervening days during which no does is applied. Suitable compositions may comprise from 0.001% to 10% w/w, for example, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation. Formulations suitable for topical administration include liquid or

semi-liquid preparations suitable for penetration through the skin (*e.g.*, liniments, lotions, ointments, creams, or pastes) and drops suitable for administration to the eye, ear, or nose.

The pharmaceutical compositions may also be prepared in a solid form (including granules, powders or suppositories). The pharmaceutical compositions may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc. Solid dosage forms for oral administration may include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound may be admixed with at least one inert diluent such as sucrose, lactose, or starch. Such dosage forms may also comprise, as in normal practice, additional substances other than inert diluents, *e.g.*, lubricating agents such as magnesium stearate. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings. Liquid dosage forms for oral administration may include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs containing inert diluents commonly used in the art, such as water. Such compositions may also comprise adjuvants, such as wetting sweetening, flavoring, and perfuming agents.

Pharmaceutical compositions comprising a nucleic acid or polypeptide of the present invention may take any of several forms and may be administered by any of several routes. In preferred embodiments, the compositions are administered via a parenteral route (intradermal, intramuscular or subcutaneous) to induce an immune response in the host. Alternatively, the composition may be administered directly into a lymph node (intranodal) or tumor mass (i.e., intratumoral administration). For example, the dose could be administered subcutaneously at days 0, 7, and 14. Suitable methods for immunization using compositions comprising TAs are known in the art, as shown for p53 (Hollstein et al., 1991), p21-ras (Almoguera et al., 1988), HER-2 (Fendly et al., 1990), the melanoma-associated antigens (MAGE-1; MAGE-2) (van der Bruggen et al., 1991), p97 (Hu et al., 1988), melanoma-associated antigen E (WO 99/30737) and carcinoembryonic antigen (CEA) (Kantor et al., 1993; Fishbein et al., 1992; Kaufman et al., 1991), among others.

Preferred embodiments of administratable compositions include, for example, nucleic acids or polypeptides in liquid preparations such as suspensions, syrups, or elixirs. Preferred injectable preparations include, for example, nucleic acids or polypeptides suitable for parental, subcutaneous, intradermal, intramuscular or intravenous administration such as sterile suspensions or emulsions. For example, a recombinant poxvirus may be in admixture with a

suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like. The composition may also be provided in lyophilized form for reconstituting, for instance, in isotonic aqueous, saline buffer. In addition, the compositions can be co-administered or sequentially administered with other antineoplastic, anti-tumor or anti-cancer agents and/or with agents which reduce or alleviate ill effects of antineoplastic, anti-tumor or anti-cancer agents.

A kit comprising a composition of the present invention is also provided. The kit can include a separate container containing a suitable carrier, diluent or excipient. The kit can also include an additional anti-cancer, anti-tumor or antineoplastic agent and/or an agent that reduces or alleviates ill effects of antineoplastic, anti-tumor or anti-cancer agents for co- or sequential-administration. Additionally, the kit can include instructions for mixing or combining ingredients and/or administration.

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

EXAMPLES

Example 1

AAC2 Tumor Associated Antigen

A version of the AAC2 coding sequence (AAC2-1) was provided by a collaborator and found to have high sequence similarity to a murine bcl-6-associated zinc finger protein ("BAZF"). Based on this sequence information, PCR primers were designed as shown below:

CACCATGGGT TCCCCCGCCG CCCCGGA (forward primer; **SEQ ID NO.: 6**)

CTAGGGCCCC CCGAGAATGT GGTAGTGCAC TTT (reverse primer; **SEQ ID NO.: 7**)

RNA was isolated from confluent HUVEC (BioWhittacker; Cat. No. CC2517, Lot No. 1F0141) cultures using Trizol™ as indicated by the manufacturer (Life Technologies, Inc., Cat. No. 15596). High fidelity RT-PCR was then performed using the forward and reverse primers (24 cycles at 94 degrees, 2 min.; 94 degrees, 30 sec; 56.8 degrees, 30 sec; 68 degrees, 1 min 40 sec; cycle 25 is 68 degrees, 7 min) resulting in the isolation of a 1,447 base pair cDNA. The cDNA was cloned into the pEF6-TOPO eukaryotic expression plasmid and termed "pEF6-hAAC2-2". The cDNA pEF6-hAAC2-2 was sequenced using four primers and aligned to the sequence of AAC2-1 and murine BAZF (**Fig. 1**). As shown therein, AAC2-2 is missing the serine residue (S) found at position 245 in AAC2-1. Secondly, a stretch of 17 amino acids at positions 298 to 316 (SEFFSCQNCEAVAGCSS) of AAC2-2 showed only 11.8% sequence

identity with amino acids 298-316 of AAC2-1 (**Fig. 1**). Interestingly, the stretch of 17 amino acids between positions 298 and 316 is 100% identical with murine BAZF suggesting that this may be critical for transcription factor function along with the long stretch of serines (zinc finger). AAC2-2 was then cloned into the pcDNA3.1-zeo eukaryotic expression plasmid ("pcDNA3.1-hAAC2-2").

Example 2

Human T-cell Reactivity Against AAC-2 Peptides

Using the AAC2-2 amino acid sequence, a library of 9-mer peptides predicted to bind to HLA-A-0201 was constructed (**Table III**; "N" indicates the sequence is not found within the mouse homolog, while "Y" indicates the sequence is found within the mouse homolog). Twenty-three of the peptides were dissolved in DMSO at 10 mg/ml (**Table IV**) and used in human PBMC cultures to test for their ability to elicit CD8 and CD4 αβ T-cell responses *in vitro*.

Table III: ***Predicted HLA-A-0201-binding nonamer peptides of human AAC2-2***

Designation	Sequence	Position in Protein	SEQ ID NO.
CLP-2954	RLSPTAATV	AAC2(256-264)	**8**
CLP-2955	SIFRGRAGV	AAC2(65-73)	**9**
CLP-2956	DVLGNLNEL	AAC2(23-31)	**10**
CLP-2957	GVGVDVLSL	AAC2(72-80)	**11**
CLP-2958	LLTSQAQDT	AAC2(277-285)	**12**
CLP-2959	VLNSQASQA	AAC2(201-209)	**13**
CLP-2960	VQFKCGAPA	AAC2(264-272)	**14**
CLP-2961	GQPCPQARL	AAC2(219-227)	**15**
CLP-2962	GAHRGLDSL	AAC2(312-320)	**16**
CLP-2963	GAPASTPYL	AAC2(269-277)	**17**
CLP-2964	VVQACHRFI	AAC2(123-131)	**18**
CLP-2965	PLGISLRPL	AAC2(137-145)	**19**
CLP-2966	PLRAHKAVL	AAC2(48-56)	**20**
CLP-2967	FVQVAHLRA	AAC2(394-402)	**21**
CLP-2968	APLLDFMYT	AAC2(90-98)	**22**
CLP-2969	RAGVGVDVL	AAC2(70-78)	**23**
CLP-2970	CETCGSRFV	AAC2(387-395)	**24**
CLP-2971	ATAPAVLAA	AAC2(106-114)	**25**
CLP-2972	SRFVQVAHL	AAC2(392-400)	**26**
CLP-2973	CNWKKYKYI	AAC2(192-200)	**27**
CLP-2974	SPAAPEGAL	AAC2(3-11)	**28**
EC-1	ALGYVREFT	AAC2(10-18)	**29**
EC-3	RLRGILTDV	AAC2(32-40)	**30**
EC-4	GILTDVTLL	AAC2(35-43)	**31**
EC-5	ILTDVTLLV	AAC2(36-44)	**32**
EC-6	TLLVGGQPL	AAC2(41-49)	**33**
EC-9	FMYTSRLRL	AAC2(95-103)	**34**
EC-10	RLSPATAPA	AAC2(102-110)	**35**
EC-11	AVLAAATYL	AAC2(110-118)	**36**
EC-12	ATYLQMEHV	AAC2(115-123)	**37**
EC-13	LQMEHVVQA	AAC2(118-126)	**38**
EC-21	QVAHLRAHV	AAC2(390-398)	**39**
EC-22	HLQTLKSHV	AAC2(418-426)	**40**
EC-24	VVQACHRFI	AAC2(123-131)	**41**

Using GM-CSF and IL-4, dendritic cells (DC) were generated from peripheral blood monocytes of blood donors expressing HLA-A-0201. DC were pulsed with the different pools of 9-mer AAC2-2 peptides shown in **Table IV.**

Table IV: ***AAC2-2 Peptide Groups***

Group #	Peptide No.	Sequences	Positions in Protein
1	CLP 2954	RLSPTAATV	AAC2(256-264)
	CLP 2956	DVLGNLNEL	AAC2(23-31)
	CLP 2957	GVGVDVLSL	AAC2(72-80)
2	CLP 2959	VLNSQASQA	AAC2(201-209)
	CLP 2960	VQFKCGAPA	AAC2(264-272)
	CLP 2963	GAPASTPYL	AAC2(269-277)
3	CLP 2964	VVQACHRFI	AAC2(123-131)
	CLP 2968	APLLDFMYT	AAC2(90-98)
4	CLP 2971	ATAPAVLAA	AAC2(106-114)
	CLP 2973	CNWKKYKYI	AAC2(192-200)
5	EC 1	ALGYVREFT	AAC2(10-18)
	EC 3	RLRGILTDV	AAC2(32-40)
	EC 3	GILTDVTLL	AAC2(35-43)
6	EC 5	ILTDVTLLV	AAC2(36-44)
	EC 6	TLLVGGQPL	AAC2(41-49)
	EC 9	FMYTSRLRL	AAC2(95-103)
7	EC 10	RLSPATAPA	AAC2(102-110)
	EC 11	AVLAAATYL	AAC2(110-118)
	EC 12	ATYLQMEHV	AAC2(115-123)
8	EC 13	LQMEHVVQA	AAC2(118-126)
	EC 21	QVAHLRAHV	AAC2(390-398)
9	EC 22	HLQTLKSHV	AAC2(418-426)
	EC 24	VVQACHRFI	AAC2(123-131)

These DC were used to stimulate autologous T-cell-enriched PBMC preparations. The T cells were re-stimulated with autologous PBMC and then re-stimulated with CD40-ligand-activated autologous B cells. After the third and fourth round of stimulation with each peptide pool, ELISPOT analysis for IFN-γ production indicated that the T cells responded most strongly

to one of the pools of AAC2-2 peptides (peptide group 6; **Fig. 2A**). Peptide group 6 includes the following peptides: ILTDVTLLV (aa 36-44), TLLVGGQPL (aa 41-49), and FMYTSRLRL (aa 95-103). Flow cytometric analysis (FACS) showed that the lymphocytes from this peptide-specific line consisted of >50% CD8 T cells with a memory ($CD45RO^+$) phenotype. Very few cells (<2%) were stained with anti-CD56 antibodies, indicating that the observed IFN-γ production was not due to NK cell activity.

Analysis of CTL activity from this peptide pool-specific T-cell line also demonstrated that the activated T cells were capable of killing peptide-loaded TAP-deficient T2 cells in an HLA-A-0201-restricted fashion (**Fig. 2B**). This analysis also revealed that ILTDVTLLV was a dominant peptide that stimulated the majority of the peptide-specific CTL activity. Thus, it was determined that AAC2-2 peptides are immunogenic in the human immune system.

EXAMPLE 3

Immunogenicity of AAC2-2 in vivo

Using DNA immunization into HLA-A2-Kb transgenic mice, it was found that the AAC2-2 protein is processed into immunogenic peptides and can elicit an HLA-A-0201-restricted T-cell response *in vivo*. Mice were immunized on day 1 by injection with pEF6-hAAC2-2 and boosted with the same plasmid at day 21. Lymphocytes were harvested from immunized mice 21 days after boosting and re-stimulated *in vitro* with the different groups of AAC2-2 peptides shown in **Table IV**. Peptide-specific effector T-cell function towards these peptides was found using IFN-γ ELISPOT analysis (**Fig. 3**). It was found that the same pool of peptides (group 6) previously shown to be strongly immunogenic in human PBMC cultures also elicited significant reactivity by T cells after DNA vaccination (**Fig. 3**). Thus, the AAC2 gene product administered as a DNA-based vaccine is immunogenic *in vivo* and elicits a strong cell-mediated immune response characterized by the activation of CTL activity.

EXAMPLE 4

Therapeutic AAC2-2 Vaccine

Therapeutic vaccination against the AAC2-2 gene product using the pEF6-hAAC2-2 DNA vaccine was found to completely block the growth of a solid tumor. Groups of eight C57BL/6 mice were subcutaneously challenged with 10^4 B16F10 melanoma cells, a vigorous and relatively non-immunogenic tumor cell line. The mice were then immunized at weekly intervals starting at 6 days after tumor challenge. Control mice (eight per group) treated either with a plasmid encoding the flu-NP protein or saline alone all developed large tumors. In contrast, all the mice (8/8) immunized with pEF6-hAAC2-2 had no detectable tumor over a 50-day period (**Fig. 4**). All mice remained tumor-free through 80 days (data not shown). **Fig. 5** plots the survival of mice treated with the different DNA vectors shown after melanoma implantation showing again the complete effectiveness of AAC2-2 vaccination in protecting mice against melanoma growth. No adverse health effects have been observed as a result of immunization with the human AAC2-2 gene-encoding vector (immunized mice were as active as control mice and showed no weight loss).

As shown in **Figs. 4** and **5**, vaccination with a plasmid encoding the human VEGFR-2 (pBLAST-hflk1) did not protect tumor-challenged mice. In fact, the tumors grew even more rapidly in these mice. Analysis of sera from mice vaccinated with the pBLAST-hflk1 plasmid by ELISA found that IgG against the VEGFR-2 protein is induced in significant titres (data not shown). These results suggest that an antibody-based immune response directed against VEGFR-2 may not be not effective in preventing angiogenesis and solid tumor growth.

Inhibition of melanoma solid tumor growth in C57BL/6 mice immunized with pEF6-hAAC2-2 correlates with an immune response against the protein (**Fig. 6**). Immunization of C57BL/6 mice was performed as described above. Spleen cells from immunized mice were re-stimulated with the same peptide pools used in experiments with HLA-A2-Kb transgenic mice (**Table III**). A significant number of peptides cross-react on C57BL/6 class I MHC (Kb and Db molecules). Two pools of peptides in particular (group 1 and group 5) were found to elicit strong effector cell activity in the IFN-γ ELISPOT assays (**Fig. 6**). All of the peptides in these groups are also identical to the corresponding sequence in the murine BAZF protein. These results strongly suggest that immunization with the human AAC2-2 activates an immune response against its murine orthologue BAZF in mice and can inhibit tumor angiogenesis as a result. These results are from a single experiment, and not all experiments showed these results.

Example 5

BFA4 Tumor Antigen

The BFA4 sequence was found to be the "trichorhinophalangeal syndrome 1" (TRPS-1) gene (Genebank ID #6684533; Momeniet et al, Nature Genetics, 24(1), 71-74,2000), a known transcription factor with no function attributed previously in any form of cancer. The BFA4 cDNA sequence is shown in **Fig. 7** (**SEQ ID NO.: 23**) and the deduced amino acid sequence is shown in **Fig. 8** (**SEQ ID NO.: 24**).

A. BFA4 Peptides and Polyclonal Antisera

For monitoring purposes, rabbit anti-BFA4 polyclonal antibodies were generated. Six peptides (22-mers) were designed and synthesized to elicit antibody response to BFA4, as shown below:

CLP 2589	MVRKKNPPLRNVASEGEGQILE	BFA4 (1-22)
CLP 2590	SPKATEETGQAQSGQANCQGLS	BFA4 (157-178)
CLP 2591	VAKPSEKNSNKSIPALQSSDSG	BFA4 (371-392)
CLP 2592	NHLQGSDGQQSVKESKEHSCTK	BFA4 (649-670)
CLP 2593	NGEQIIRRRTRKRLNPEALQAE	BFA4 (940-961)
CLP 2594	ANGASKEKTKAPPNVKNEGPLNV	BFA4 (1178-1199)

Rabbits were immunized with the peptides, serum was isolated, and the following antibody titers were observed:

Rabbit #	**Peptide**	Titer (Bleed 2)	Titer (Final Bleed)
1,2	CLP2589	800000, 1600000	2560000, 2560000
3,4	CLP2590	12800, 6400	40000, 40000
5,6	CLP2591	400000, 400000	320000, 320000
7,8	CLP2592	25600, 12800	80000, 40000
9,10	CLP2593	3200000, 51200	2560000, 160000
11,12	CLP2594	409600, 409600	320000, 320000

These peptides were also modified by coupling with KLH peptides to enhance immune responses as shown below:

BFA4(1-22)	KLH-MVRKKNPPLRNVASEGEGQILE	(CLP-2589)
BFA4(157-178)	KLH-SPKATEETGQAQSGQANCQGLS	(CLP-2590)
BFA4(371-392)	KLH-VAKPSEKNSNKSIPALQSSDSG	(CLP-2591)
BFA4(649-670)	KLH-NHLQGSDGQQSVKESKEHSCTK	(CLP-2592)
BFA4(940-961)	KLH-NGEQIIRRRTRKRLNPEALQAE	(CLP-2593)
BFA4(1178-1200)	KLH-ANGASKEKTKAPPNVKNEGPLNV	(CLP-2594)

The pcDNA3.2BFA4 (3.6 mg) was also used for DNA immunization to generate polyclonal sera in chickens.

B. Cloning of BFA4

Complete cDNA sequence for BFA4 is ~10kb and gene is expressed in BT474 ductal carcinoma cells. Primers 7717 (forward primer) and 7723 (reverse primer) were designed to amplify full-length BFA4 gene by amplification of 4kb, 7kb or 10kb products by RT-PCR.

Primer 7717: BFA4-BamH1/F1 (5' end forward) with Kozak:

5' CGGGATCCACCATGGTCCGGAAAAAGAACCCC 3'(BamHI for DNA3.1, MP76)

Primer 7723: BFA4-BamHI /R1 (3' end reverse 4kb):

5' CGGGATCCCTCTTTAGGTTTTCCATTTTTTTCCAC 3' (BamHI for DNA3.1, MP76)

Ten mg of total RNA isolated and frozen in different batches from BT-474 cells using Trizol as indicated by the manufacturer (Gibco BRL) was used in RT-PCR to amplify the BFA4 gene. RT-PCR conditions were optimized using Taq Platinum High Fidelity enzyme, OPC (Oligo Purification Cartridge; Applied Biosystems) purified primers and purified total RNA/polyA mRNA (BT 474 cells). Optimization resulted in a 4.0kb fragment as a single band.

To re-amplify the BFA4 sequence, mRNA was treated with DNase per manufacturers' instructions (Gibco BRL). The 4kb DNA was reamplified using PCR using primers 7717 and 7723 primers (10pmole/microlitre) and Taq Platinum High Fidelity polymerase (GIBCO BRL) enzyme. Thermocycler conditions for both sets of reactions were as under: 94°C (2 min), followed by 30 cycles of 94°C (30 sec), 52°C (30sec), 67°C (4 min) and 67°C (5 min) and finally 40°C for 10 min. Three BFA4 clones were identified after pCR2.1/TOPO-TA cloning.

Several mutations were identified during analysis of the BFA4 sequence. To correct these sequences, the BamHI/XhoI fragment (5') of the BFA4 gene from clone JB-3552-1-2 (pCR2.1/TOPO/ BFA4) was exchanged with the XhoI/BamHI fragment (3') of the BFA4 gene from clone JB-3552-1-4 (pCR2.1/TOPO/BFA4). This recombined fragment was then ligated into pMCS5 BamHI/CAP. Clone JB-3624-1-5 was generated and found to contain the correct sequence.

Nucleotide 344 of the isolated BFA4 clone was different from the reported sequence (C in BFA4, T in TRPS-1). The change resulted in a phe to ser amino acid change. To change this sequence to the reported sequence, the EcoRI/BglII fragment (5') of the BFA4 gene from clone

JB-3552-1-2 (pCR2.1/TOPO/BFA4) was subcloned into pUC8:2 to generate clone JB-3631-2. This clone was used as a template for Quickchange (Stratagene) mutagenesis to change amino acid 115 of the BFA4 protein from a serine to a phenylalanine as in the TRPS1 protein. The selected clone was JB-3648-2-3. Mutagenesis was also repeated with pMCS5 BFA4 (BT474) as a template for Quickchange (Stratagene) mutagenesis to change amino acid 115 of the BFA4 protein from a serine to a phenylalanine as in the TRPS1 protein. Several clones were found to be correct by DNA sequencing and one of the clones (JB-3685-1-18) was used for further subcloning.

JB-3685-1-18 was then used to subclone the BFA4 coding sequence into the *Bam*HI sites of four different expression vectors: 1) the poxviral (NYVAC) vector pSD554VC (COPAK/H6; JB-3707-1-7); 2) pcDNA3.1/Zeo (+) (JB-3707-3-2); 3) pCAMycHis (JB-3707-5-1); and, 4) Semiliki Forest virus alphaviral replicon vector pMP76 (JB-3735-1-23). The BFA4 coding sequence within JB-3707-1-7, JB-3707-5-1, and JB-3735-1-23 was confirmed by DNA sequencing.

A stop codon was introduced near the end of the cloned sequence in the pcDNA3.1/Zeo/BFA4 construct (JB-3707-3-2). A unique EcoR1 site was opened and filled in to introduce a stop codon in-frame with BFA4 coding sequence. Several putative clones were identified by the loss of EcoR1 site, however three clones (JB-3756-1-2; JB-3756-3-1; and JB-3756-4-1) were sequenced. All three were found to be correct in the area of the fill-in. Clone JB-3756-3-1 identified as having the correct sequence and orientation.

Myc and myc/his tags (Evans et al, 1985) were introduced using oligonucleotides, which were annealed and ligated into the pcDNA3.1/Zeo/BFA4 construct (JB-3707-3-2) at the EcoRI /EcoRV sites. Several clones were obtained for these constructs. Three clones having the correct sequences and orientations were obtained: 1) PcDNA3.1/Zeo/BFA4/myc-tag (JB-3773-1-2); 2) PcDNA3.1/Zeo/BFA4/mychis-tag (JB-3773-2-1); and, 3) PcDNA3.1/Zeo/BFA4/mychis-tag (JB-3773-2-2).

C. Expression of BFA4

1. Expression from poxviral vectors

The pSD554VC (COPAK/H6; JB-3707-1-7) vector was used to generate NYVAC-BFA4 virus. *In vitro* recombination was performed with plasmid COPAK/H6/BFA4 and NYVAC in RK13/CEF cells. NYVAC-BFA4 (vP2033-NYVAC-RK13) was generated and amplified to P3 level after completion of three enrichments with final stock concentrations of 1.12 x 10^9/ml

(10ml). Vero cells were infected with NYVAC-BFA4 at an M.O.I. of 0.5 pfu/cell. Lysates and media were harvested 24h post-infection to confirm expression of BFA4 protein. One-twentieth of the concentrated media and 1/40 of the lysate were loaded onto a western blot and incubated with rabbit antisera against the BFA4 peptides CLP 2589, 2591, 2598 and 2594 (see above for peptide sequences and preparation of anti-BFA4 antisera). An approximate 120kD band was detected in both the lysate and the concentrated media of NYVAC-BFA4-infected Vero cells which was not evident in either Vero control cells ("mock-infected"), Vero cells infected with the parental NYVAC virus, or concentrated media.

2. Expression from pcDNA3.1-based vectors

Transient transfection studies were performed to verify expression of BFA4 from the pcDNA-based vectors and to analyze quality of polyclonal sera raised against BFA4 peptides. The following constructs were used to study expression of BFA4 gene: pcDNA 3.1 zeo^R/BFA4, pMP76/BFA4, pcDNA 3.1 zeo^R/ BFA4/Myc tag and pcDNA 3.1 zeo^R/ BFA4/MycHis tag. BFA4 expression plasmids (5μg and 10 μg) were co-transfected with pGL3 Luciferase (1□g) (Promega) with the Gene porter reagent (Gene Therapy Systems) as the transfection reagent. At 48h post-transfection, whole cell extract was prepared by scraping cells in cell lysis reagent (200μl) and 1 cycle of freeze-thaw (–20°C freeze, 37°C thaw). Transfection efficiency was quantitated by analyzing expression of the luciferase reporter gene by measuring Relative Luciferase Units (RLU) in duplicate. Similar RLU values were obtained in the samples co-transfected with luciferase construct in the presence and absence of BFA4 expression vectors. There was no significant difference observed in toxicity or RLU values with differential amount (5μg and 10 μg) of BFA4 expression vectors. Preliminary western blot analysis using alkaline phosphatase system with the CHOK1 cell extracts (pCDNA3.1 /zeo/ BFA4/MycHisTag) and an anti-BFA4 polyclonal antisera, revealed a band at approximately 120kDa band in extracts of BFA4 vector-transfected cells.

A stable transfection study was initiated to obtain stable clones of BFA4 expressing COS A2 cells. These cells are useful for *in vitro* stimulation assays. pcDNA 3.1 zeo^R/BFA4 (2.5μg and 20 μg), and pcDNA 3.1 zeo ^{R}/ BFA4/MycHis tag (2.5μg) were used to study expression of BFA4). pGL3 Luciferase (2.5μg) was used as a control vector to monitor transfection efficiency. The Gene porter reagent was used to facilitate transfection of DNA vectors. After 48h post-transfection, whole cell extract were prepared by scraping cells in the cell lysis reagent (200μl)

and 1 cycle of freeze-thaw at –20°C/37°C for first experiment. Transfected cells obtained from the second experiment were trypsinized, frozen stock established and cells were plated in increasing concentrations of Zeocin (0, 250, 500, 750 and 1000μg/ml). Non-transfected CosA2cells survived at 60-80 % confluency for three weeks at 100μg/ml (Zeocin) and 10% confluency at 250μg/ml (Zeocin). However, after three weeks, at higher drug concentration (500-1000μg/ml), live cells were not observed in the plates containing non-transfected cells and high Zeocin concentration (500-1000μg/ml).

Several Zeocin-resistant clones growing in differential drug concentrations (Zeocin-250, 500, 750 and 1000μg/ml) were picked from 10 cm plates after three weeks. These clones were further expanded in a 3.5 cm plate(s) in the presence of Zeocin at 500, 750 and 1000 μg/ml. Frozen lots of these clones were prepared and several clones from each pool (pcDNA 3.1 zeo^R/BFA4, and pcDNA 3.1 zeo^R/ BFA4/MycHis tag) were expanded to T75 cm^2 flasks in the presence of Zeocin at 1mg/ml. Five clones from each pool (pcDNA 3.1 zeo^R/BFA4, and pcDNA 3.1 zeo^R/ BFA4/MycHis tag) were expanded to T75 cm^2 flasks in the presence of Zeocin at 1mg/ml. Cells are maintained under Zeocin drug (1mg/ml) selection. Six clones were used in BFA4 peptide-pulsed target experiment, and two clones were found to express BFA4 at a moderate level by immunological assays. The non-adherent cell lines K562A2 and EL4A2 were also transfected with these vectors to generate stable cell lines.

3. Prokaryotic expression vector

The BamHI –Xho-1 fragment (1.5 Kbp) fragment encoding N-terminal 54kDa BFDA4 from pCDNA3.1/BFA4 was cloned into pGEX4T1-6His (Veritas) plasmid. This vector contains the tac promoter followed by the N-terminal glutathione S-transferase (GST~26kDa) and a hexahistidine tag to C terminus of the GST fusion protein.

The BFA4-N54 expression plasmid was transformed into BL21 cells and grown at 25°C in antibiotic selection medium (2L culture) to an OD (600nm) and thereafter induced with 1mM IPTG. GST-BFA4-N54 was found to be soluble protein. Clarified extract of the soluble fraction was adsorbed batchwise to glutathione-Sepharose 4B and eluted with 10mM reduced glutathione. Fractions were analyzed after estimation of protein concentration and TCA precipitation. Specific polypeptide of Mr=85kDa in the eluate was confirmed by SDS-PAGE. The recombinant protein was purified by gluathione-Sepharose was absorbed on a NiNTA column for further purification. The bound protein was eluted with 0.25M imidazole. The protein was dialyzed versus TBS containing 40% Glycerol, resulting in 4.5 mg GST-BFA4-N54-6 His (N terminus

BFA4 protein) protein. Expression of BFA4 was confirmed using the rabbit anti-BFA4 polyclonal antibody by western blot.

D. Anti-BFA4 immune responses

1. BFA4 peptides

In addition to genetic immunization vectors for BFA4, immunological reagents for BFA4 have been generated. A library of 100 nonamer peptides spanning the BFA4 gene product was synthesized. The peptides were chosen based on their potential ability to bind to HLA-A*0201. **Table V** lists 100 nonamer peptide epitopes for HLA-A*0201 from the BFA4 protein tested (see below):

PEPTIDE DESIGNATION	SEQUENCE	POSITION IN PROTEIN
CLP- 2421	MVRKKNPPL	BFA4 (1-9)I"
CLP- 2422	KKNPPLRNV	BFA4 (4-12)I"
CLP- 2423	VASEGEGQI	BFA4 (12-20)I"
CLP- 2424	QILEPIGTE	BFA4 (19-27)I"
CLP- 2425	RNMLAFSFP	BFA4 (108-116)I"
CLP- 2426	NMLAFSFPA	BFA4 (109-117)I"
CLP- 2427	MLAFSFPAA	BFA4 (110-118)I"
CLP- 2428	FSFPAAGGV	BFA4 (113-121)I"
CLP- 2429	AAGGVCEPL	BFA4 (117-125)I"
CLP- 2430	SGQANCQGL	BFA4 (170-178)I"
CLP- 2431	ANCQGLSPV	BFA4 (172-180)I"
CLP- 2432	GLSPVSVAS	BFA4 (176-184)I"
CLP- 2433	SVASKNPQV	BFA4 (181-189)I"
CLP- 2434	RLNKSKTDL	BFA4 (196-204)I"
CLP- 2435	NDNPDPAPL	BFA4 (207-215)I"
CLP- 2436	DPAPLSPEL	BFA4 (211-219)I"
CLP- 2437	ELQDFKCNI	BFA4 (218-216)I"
CLP- 2438	GLHNRTRQD	BFA4 (249-257)I"
CLP- 2439	ELDSKILAL	BFA4 (259-267)I"
CLP- 2440	KILALHNMV	BFA4 (263-271)I"
CLP- 2441	ALHNMVQFS	BFA4 (266-284)I"
CLP- 2442	VNRSVFSGV	BFA4 (282-290)I"
CLP- 2443	FSGVLQDIN	BFA4 (287-295)I"
CLP- 2444	DINSSRPVL	BFA4 (293-301)I"
CLP- 2445	VLLNGTYDV	BFA4 (300-308)I"
CLP- 2446	FCNFTYMGN	BFA4 (337-345)I"
CLP- 2447	YMGNSSTEL	BFA4 (342-350)I"
CLP- 2448	FLQTHPNKI	BFA4 (354-362)I"
CLP- 2449	KASLPSSEV	BFA4 (363-371)I"
CLP- 2450	DLGKWQDKI	BFA4 (393-401)I"
CLP- 2451	VKAGDDTPV	BFA4 (403-411)I"
CLP- 2452	FSCESSSSL	BFA4 (441-449)I"
CLP- 2453	KLLEHYGKQ	BFA4 (450-458)I"
CLP- 2454	GLNPELNDK	BFA4 (466-474)I"
CLP- 2455	GSVINQNDL	BFA4 (478-486)I"
CLP- 2456	SVINQNDLA	BFA4 (479-487)I"
CLP- 2457	FCDFRYSKS	BFA4 (527-535)I"
CLP- 2458	SHGPDVIVV	BFA4 (535-543)I"
CLP- 2459	PLLRHYQQL	BFA4 (545-553)I"
CLP- 2460	GLCSPEKHL	BFA4 (570-578)I"
CLP- 2461	HLGEITYPF	BFA4 (577-585)I"
CLP- 2462	LGEITYPFA	BFA4 (578-586)I"
CLP- 2463	HCALLLLHL	BFA4 (594-602)I"
CLP- 2464	ALLLLHLSP	BFA4 (596-604)I"
CLP- 2465	LLLLHLSPG	BFA4 (597-605)I"
CLP- 2466	LLLHLSPGA	BFA4 (598-606)I"
CLP- 2467	LLHLSPGAA	BFA4 (599-607)I"
CLP- 2468	FTTPDVDVL	BFA4 (621-629)I"
CLP- 2469	TTPDVDVLL	BFA4 (622-630)I"
CLP- 2470	VLLFHYESV	BFA4 (628-636)I"
CLP- 2471	FITQVEEEI	BFA4 (673-681)I"
CLP- 2472	FTAADTQSL	BFA4 (699-707)I"
CLP- 2473	SLLEHFNTV	BFA4 (706-714)I"

PEPTIDE DESIGNATION	SEQUENCE	POSITION IN PROTEIN
CLP- 2474	STIKEEPKI	BFA4 (734-742)1"
CLP- 2475	KIDFRVYNL	BFA4 (741-749)1"
CLP- 2476	NLLTPDSKM	BFA4 (748-756)1"
CLP- 2479	VTWRGADIL	BFA4 (792-800)1"
CLP- 2480	ILRGSPSYT	BFA4 (799-807)1"
CLP- 2481	YTQASLGLL	BFA4 (806-814)1"
CLP- 2482	ASLGLLTPV	BFA4 (809-817)1"
CLP- 2483	GLLTPVSGT	BFA4 (812-820)1"
CLP- 2484	GTQEQTKTL	BFA4 (819-827)1"
CLP- 2485	KTLRDSPNV	BFA4 (825-833)1"
CLP- 2486	HLARPIYGL	BFA4 (837-845)1"
CLP- 2487	PIYGLAVET	BFA4 (841-849)1"
CLP- 2488	LAVETKGFL	BFA4 (845-853)1"
CLP- 2489	FLQGAPAGG	BFA4 (852-860)1"
CLP- 2490	AGGEKSGAL	BFA4 (858-866)1"
CLP- 2491	GALPQQYPA	BFA4 (864-872)1"
CLP- 2492	ALPQQYPAS	BFA4 (865-873)1"
CLP- 2493	FCANCLTTK	BFA4 (895-903)1"
CLP- 2494	ANGGYVCNA	BFA4 (911-919)1"
CLP- 2495	NACGLYQKL	BFA4 (918-926)1"
CLP- 2496	GLYQKLHST	BFA4 (921-929)1"
CLP- 2497	KLHSTPRPL	BFA4 (925-933)1"
CLP- 2498	STPRPLNII	BFA4 (928-936)1"
CLP- 2499	RLNPEALQA	BFA4 (952-960)1"
CLP- 2500	VLVSQTLDI	BFA4 (1020-1028)1"
CLP- 2501	DIHKRMQPL	BFA4 (1027-1035)1"
CLP- 2502	RMQPLHIQI	BFA4 (1031-1039)1"
CLP- 2503	YPLFGLPFV	BFA4 (1092-1100)1"
CLP- 2504	GLPFVHNDF	BFA4 (1096-1104)1"
CLP- 2505	FVHNDFQSE	BFA4 (1099-1107)1"
CLP- 2506	SVPGNPHYL	BFA4 (1120-1128)1"
CLP- 2507	GNPHYLSHV	BFA4 (1123-1131)1"
CLP- 2508	HYLSHVPGL	BFA4 (1126-1134)1"
CLP- 2509	YVPYPTFNL	BFA4 (1141-1149)1"
CLP- 2510	FNLPPHFSA	BFA4 (1147-1155)1"
CLP- 2511	NLPPHFSAV	BFA4 (1148-1156)1"
CLP- 2512	SAVGSDNDI	BFA4 (1154-1162)1"
CLP- 2513	KNEGPLNVV	BFA4 (1192-1200)1"
CLP- 2514	TKCVHCGIV	BFA4 (1215-1223)1"
CLP- 2515	CVHCGIVFL	BFA4 (1217-1225)1"
CLP- 2516	CGIVFLDEV	BFA4 (1220-1228)1"
CLP- 2517	FLDEVMYAL	BFA4 (1224-1232)1"
CLP- 2518	VMYALHMSC	BFA4 (1228-1236)1"
CLP- 2519	FQCSICQHL	BFA4 (1243-1251)1"
CLP- 2520	GLHRNNAQV	BFA4 (1265-1273)

The peptide library was pooled into separate groups containing 7-10 different peptides for immunological testing as shown in **Table VI** (see below). In addition to a peptide library spanning BFA4, a recombinant protein spanning the N-terminal 300 amino acids (positions 1-300) has been synthesized and purified from *E. coli*.

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PEPTIDE GROUP	PEPTIDE NUMBER	SEQUENCE
1	CLP- 2421	MVRKKNPPL
	CLP- 2422	KKNPPLRNV
	CLP- 2423	VASEGEGQI
	CLP- 2424	QILEPIGTE
	CLP- 2425	RNMLAFSFP
	CLP- 2426	NMLAFSFPA
	CLP- 2427	MLAFSFPAA
	CLP- 2428	FSFPAAGGV
	CLP- 2429	AAGGVCEPL
	CLP- 2430	SGQANCQGL
2	CLP- 2431	ANCQGLSPV
	CLP- 2432	GLSPVSVAS
	CLP- 2433	SVASKNPQV
	CLP- 2434	RLNKSKTDL
	CLP- 2435	NDNPDPAPL
	CLP- 2436	DPAPLSPEL
	CLP- 2437	ELQDFKCNI
	CLP- 2438	GLHNRTRQD
	CLP- 2439	ELDSKILAL
	CLP- 2440	KILALHNMV
3	CLP- 2441	ALHNMVQFS
	CLP- 2442	VNRSVFSGV
	CLP- 2443	FSGVLQDIN
	CLP- 2444	DINSSRPVL
	CLP- 2445	VLLNGTYDV
	CLP- 2446	FCNFTYMGN
	CLP- 2447	YMGNSSTEL
	CLP- 2448	FLQTHPNKI
	CLP- 2449	KASLPSSEV
	CLP- 2450	DLGKWQDKI
4	CLP- 2451	VKAGDDTPV
	CLP- 2452	FSCESSSSL
	CLP- 2453	KLLEHYGKQ
	CLP- 2454	GLNPELNDK
	CLP- 2455	GSVINQNDL
	CLP- 2456	SVINQNDLA
	CLP- 2457	FCDFRYSKS
	CLP- 2458	SHGPDVIVV
	CLP- 2459	PLLRHYQQL
	CLP- 2460	GLCSPEKHL
5	CLP- 2461	HLGEITYPF
	CLP- 2462	LGEITYPFA
	CLP- 2463	HCALLLLHL
	CLP- 2464	ALLLLHLSP
	CLP- 2465	LLLLHLSPG
	CLP- 2466	LLLHLSPGA
	CLP- 2467	LLHLSPGAA
	CLP- 2468	FTTPDVDVL
	CLP- 2469	TTPDVDVLL
	CLP- 2470	VLLFHYESV

PEPTIDE GROUP	PEPTIDE NUMBER	SEQUENCE
6	CLP- 2471	FITQVEEEI
	CLP- 2472	FTAADTQSL
	CLP- 2473	SLLEHFNTV
	CLP- 2474	STIKEEPKI
	CLP- 2475	KIDFRVYNL
	CLP- 2476	NLLTPDSKM
	CLP- 2477	KMGEPVSES
	CLP- 2478	GLKEKVWTE
	CLP- 2479	VTWRGADIL
	CLP- 2480	ILRGSPSYT
7	CLP- 2481	YTQASLGLL
	CLP- 2482	ASLGLLTPV
	CLP- 2483	GLLTPVSGT
	CLP- 2484	GTQEQTKTL
	CLP- 2485	KTLRDSPNV
	CLP- 2486	HLARPIYGL
	CLP- 2487	PIYGLAVET
	CLP- 2488	LAVETKGFL
	CLP- 2489	FLQGAPAGG
	CLP- 2490	AGGEKSGAL
8	CLP- 2491	GALPQQYPA
	CLP- 2492	ALPQQYPAS
	CLP- 2493	FCANCLTTK
	CLP- 2494	ANGGYVCNA
	CLP- 2495	NACGLYQKL
	CLP- 2496	GLYQKLHST
	CLP- 2497	KLHSTPRPL
	CLP- 2498	STPRPLNII
	CLP- 2499	RLNPEALQA
	CLP- 2500	VLVSQTLDI
9	CLP- 2501	DIHKRMQPL
	CLP- 2502	RMQPLHIQI
	CLP- 2503	YPLFGLPFV
	CLP- 2504	GLPFVHNDF
	CLP- 2505	FVHNDFQSE
	CLP- 2506	SVPGNPHYL
	CLP- 2507	GNPHYLSHV
	CLP- 2508	HYLSHVPGL
	CLP- 2509	YVPYPTFNL
	CLP- 2510	FNLPPHFSA
10	CLP- 2511	NLPPHFSAV
	CLP- 2512	SAVGSDNDI
	CLP- 2513	KNEGPLNVV
	CLP- 2514	TKCVHCGIV
	CLP- 2515	CVHCGIVFL
	CLP- 2516	CGIVFLDEV
	CLP- 2517	FLDEVMYAL
	CLP- 2518	VMYALHMSC
	CLP- 2519	FQCSICQHL
	CLP- 2520	GLHRNNAQV

2. Immune reactivity of BFA4 peptides and generation of human effector T cells:

The BFA4 peptides were grouped into different pools of 7-10 peptides for immunological testing. Dissolved peptide pools were pulsed onto autologous HLA-A*0201 dendritic cells and used to activate autologous T-cell-enriched PBMC preparations. Activated T cells from each peptide-pool-stimulated culture were re-stimulated another 3 to 5 times using CD40L-activated autologous B-cells. IFN-γ ELISPOT analysis and assays for CTL killing of peptide-pulsed target cells was performed to demonstrate the immunogenicity of these epitopes from BFA4.

Human T cells demonstrated effector cell activity against a number of pools of peptides from the BFA4 protein, as shown by their ability to secrete IFN-γ in ELISPOT assays. These experiments were repeated after different rounds of APC stimulation resulting in the same reactive peptide groups. Peptide groups 1, 2, 4, 5, 6, 7, 8, 9, and 10 were found to be immunoreactive in these assays (**Fig. 9A**). Subsequently, these reactive peptide groups were de-convoluted in additional IFN-γ ELISPOT assays in which single peptides from each group were tested separately. The individual peptides from BFA4 peptide groups 1, 5 6, 7, 8, 9, and 10 in ELISPOT assays (**Fig. 9B**). This analysis revealed a number of individual strongly reactive peptides from the BFA4 protein recognized by human T cells. It was also observed that many of these single peptides also induced CTL activity killing peptide-loaded human T2 lymphoma cell targets. These peptides are listed in **Table VII**:

Table VII

List of highly immunoreactive peptides from BFA4

Strong IFN-γ Killing		Strong CTL Killing	
CLP 2425	**RNMLAFSFP**	CLP 2425	**RNMLAFSFP**
CLP 2426	**NMLAFSFPA**	CLP 2426	**NMLAFSFPA**
CLP 2427	**MLAFSFPAA**	CLP 2427	**MLAFSFPAA**
CLP 2461	**HLGEITYPF**		
CLP 2468	**FTTPDVDVL**	CLP 2468	**FTTPDVDVL**
CLP 2470	**VLLFHYESV**	CLP 2470	**VLLFHYYESV**
CLP 2474	**KIDFRVYNL**		
CLP 2482	**ASLGLLTPV**	CLP 2482	**ASLGLLTPV**
CLP 2486	**HLARPIYGL**	CLP 2486	**HLARPIYGL**
CLP 2495	**NACGLYQKL**	CLP 2495	**NACGLYQKL**
CLP 2497	**KLHSTPRPL**		
CLP 2499	**RLNPEALQA**	CLP 2499	**RLNPEALQA**
CLP 2503	**YPLFGLPFV**		
CLP 2509	**YVPYPTFNL**	CLP 2509	**YVPYPTFNL**
CLP 2511	**NLPPHFSAV**		
CLP 2518	**VMYALHMSC**		
CLP 2520	**GLHRNNAQV**	CLP 2520	**GLHRNNAQV**

D. Immune responses against BFA4 after immunization *in vivo*:

The pcDNA3.1/Zeo-BFA4 plasmid was used to immunize transgenic mice expressing a hybrid HLA-A*0201 α1α2 domain fused to a murine Kb α3 domain in C57BL/6 mice (A2-Kb mice). IFN-γ ELISPOT analysis using the groups of pooled peptides after DNA immunization and removal of activated spleen cells revealed a number of reactive BFA4 peptide groups. Some of these groups (especially group 7 and 8) also reacted strongly in human T-cell cultures suggesting that overlapping groups of peptides are recognized by human T cells and are naturally processed and presented on HLA-A2 after vaccination.

Vaccination experiments were also performed with the NYVAC-BFA4 and the MP76-18-BFA4 vectors in A2-Kb mice. Mice were immunized subcutaneously with 10-20μg of MP-76-18-BFA4 and 1-2 x 10^7 pfu vP2033 (NYVAC-BFA4) and boosted 28 days later with the same amounts of each vector. Re-stimulation of spleen cells from the immunized mice with the pools of BFA4 peptides revealed induction of IFN-γ production in response to BFA4 peptide groups 2, 3, 4, 5, 7, 9, and 10 in ELISPOT assays. Thus, the BFA4 gene encoded in a CMV promoter

driven eukaryotic plasmid, NYVAC, or a Semliki replicase-based DNA plasmid, were all capable of inducing T-cell responses against the BFA4 protein *in vivo*.

Example 6

BCY1 Tumor Antigen

The BCY1 gene was detected as a partial open reading frame (ORF) homologous to a nematode gene called "posterior-expressed maternal gene-3" (PEM-3) playing a role in posterior to anterior patterning in *Caenorhabtidis elegans* embryos. No previous involvement of this gene in cancer has been documented.

A. BCY1 and Amino Acid DNA Sequences

A partial DNA sequence was originally determined for BCY1. Primers, 9616SXC and 9617SXC, are derived from the BCY I partial DNA sequence and are designed to clone BCY I by RT-PCR from Calu 6 total RNA. The primers were designed such that the PCR product has BamHI sites at both ends and an ATG start codon and a Kozak sequence at the 5' end, as shown below:

```
9616SXC:   5' CAGTACGGATCCACCATGGCCGAGCTGCGCCTGAAGGGC 3'

9617SXC:   5' CCACGAGGATCCTTAGGAGAATATTCGGATGGCTTGCG 3'
```

The 1.2 Kb expected amplicon was obtained using ThermoScript RT-PCR System (Invitrogen) under optimized conditions. The PCR products from three separate RT-PCR's were digested with BamHI and respectively inserted in pcDNA3.1/Zeo(+). The resulting clones were MC50A6, MC50A8 and MC50A19 from the first RT-PCR; MC54.21 from the second RT-PCR and MC55.29; and, MC55.32 from the third RT-PCR. The following primers were utilized in sequencing the clones:

```
9620MC:      5' TAATACGACTCACTATAGGG 3'
9621MC:      5' TAGAAGGCACAGTCGAGG 3'
9618MC:      5' GAAAACGACTTCCTGGCGGGGAG 3'
9619MC:      5' GCTCACCCAGGCGTGGGGCCTC 3'
```

DNA sequencing of all six clones indicated a consensus sequence (**SEQ ID NO.: 25**), as shown in **Figs. 10A** and **10B**, having the following differences from the original partial BCY1 sequence: a C to G substitution at position 1031 resulting in an amino acid change of Ala to Gly; a GC deletion at position 1032-1034 resulting in a Thr deletion; and, an A to G substitution at

position 1177 resulting in an amino acid change of Thr to Ala. Clones MC50A8 and MC55.29 are identical to the consensus sequence. The amino acid sequence of BCY1 is shown in **Fig. 10B** and (**SEQ ID NO.: 26**).

B. Immunological reagents for BCY1 breast cancer antigen:

A library of 100 nonamer peptides spanning the BCY1 gene product was synthesized. The peptides were chosen based on their potential ability to bind to HLA-A*0201. **Table VIII** lists 100 nonamer peptide epitopes for HLA-A*0201 from the BCY1 protein tested (see below):

Table VIII

Peptide Designation	Sequence	Position in Protein
*CLP-2599	VPVPTSEHV	2
*CLP-2602	PTSEHVAEI	5
*CLP-2609	EIVGRQCKI	12
*CLP-2616	KIKALRAKT	19
*CLP-2618	KALRAKTNT	21
*CLP-2619	ALRAKTNTY	22
*CLP-2620	LRAKTNTYI	23
*CLP-2624	TNTYIKTPV	27
*CLP-2627	YIKTPVRGE	30
*CLP-2630	TPVRGEEPV	33
*CLP-2633	RGEEPVFMV	36
*CLP-2640	MVTGRREDV	43
CLP-2641	VTGRREDVA	44
*CLP-2643	GRREDVATA	46
CLP-2647	DVATARREI	50
CLP-2648	VATARREII	51
*CLP-2650	TARREIISA	53
*CLP-2651	ARREIISAA	54
*CLP-2655	IISAAEHFS	58
*CLP-2656	ISAAEHFSM	59
CLP-2657	SAAEHFSMI	60
*CLP-2659	AEHFSMIRA	62
*CLP-2663	SMIRASRNK	66
CLP-2666	RASRNKSGA	69
*CLP-2670	NKSGAAFGV	73
*CLP-2673	GAAFGVAPA	76
*CLP-2674	AAFGVAPAL	77
*CLP-2677	GVAPALPGQ	80
*CLP-2678	VAPALPGQV	81
*CLP-2680	PALPGQVTI	83
*CLP-2681	ALPGQVTIR	84
*CLP-2682	LPGQVTIRV	85
CLP-2684	GQVTIRVRV	87
*CLP-2689	RVRVPYRVV	92
*CLP-2691	RVPYRVVGL	94
*CLP-2692	VPYRVVGLV	95
*CLP-2695	RVVGLVVGP	98
*CLP-2698	GLVVGPKGA	101
*CLP-2699	LVVGPKGAT	102
*CLP-2700	VVGPKGATI	103
*CLP-2710	RIQQQTNTY	113
*CLP-2711	IQQQTNTYI	114
*CLP-2712	QQQTNTYII	115
*CLP-2713	QQTNTYIIT	116
*CLP-2718	YIITPSRDR	121
CLP-2721	TPSRDRDPV	124
CLP-2724	RDRDPVFEI	127
CLP-2731	EITGAPGNV	134
CLP-2734	GAPGNVERA	137
CLP-2738	NVERAREEI	141
CLP-2744	EEIETHIAV	147
CLP-2746	IETHIAVRT	149

Table VIII (continued)

PEPTIDE DESIGNATION	SEQUENCE	POSITION IN PROTEIN
CLP-2749	HIAVRTGKI	152
CLP-2750	IAVRTGKIL	153
CLP-2756	KILEYNNEN	159
CLP-2760	YNNENDFLA	163
CLP-2762	NENDFLAGS	165
CLP-2766	FLAGSPDAA	169
CLP-2767	LAGSPDAAI	170
CLP-2774	AIDSRYSDA	177
CLP-2777	SRYSDAWRV	180
CLP-2785	VHQPGCKPL	188
CLP-2793	LSTFRQNSL	196
CLP-2801	LGCIGECGV	204
CLP-2807	CGVDSGFEA	210
CLP-2812	GFEAPRLDV	215
CLP-2817	RLDVYYGVA	220
CLP-2819	DVYYGVAET	222
CLP-2823	GVAETSPPL	226
CLP-2825	AETSPPLWA	228
CLP-2830	PLWAGQENA	233
CLP-2833	AGQENATPT	236
CLP-2835	QENATPTSV	238
CLP-2843	VLFSSASSS	246
CLP-2857	KARAGPPGA	260
CLP-2869	PATSAGPEL	272
CLP-2870	ATSAGPELA	273
CLP-2872	SAGPELAGL	275
CLP-2879	GLPRRPPGE	282
CLP-2887	EPLQGFSKL	290
CLP-2892	FSKLGGGGL	295
CLP-2894	KLGGGGLRS	297
CLP-2899	GLRSPGGGR	302
CLP-2909	CMVCFESEV	312
CLP-2910	MVCFESEVT	313
CLP-2911	VCFESEVTA	314
CLP-2913	FESEVTAAL	316
CLP-2916	EVTAALVPC	319
CLP-2917	VTAALVPCG	320
CLP-2920	ALVPCGHNL	323
CLP-2921	LVPCGHNLF	324
CLP-2922	VPCGHNLFC	325
CLP-2927	NLFCMECAV	330
CLP-2929	FCMECAVRI	332
CLP-2933	CAVRICERT	336
CLP-2936	RICERTDPE	339
CLP-2940	RTDPECPVC	343
CLP-2945	CPVCHITAT	348
CLP-2947	VCHITATQA	350
CLP-2950	ITATQAIRI	353

Table IX shows the groups of peptides used for immunological testing:

Peptide Group	Peptide Number	Peptide Sequence
1	CLP 2887	EPLQGFSKL
	CLP 2916	EVTAALVPC
	CLP 2945	CPVCHITAT
	CLP 2673	KIKALRAKT
	CLP 2699	IISAAEHFS
	CLP 2616	RASRNKSGA
	CLP 2655	GAAFGVAPA
	CLP 2731	LVVGPKGAT
	CLP 2734	EITGAPGNV
	CLP 2666	GAPGNVERA
2	CLP 2724	ALRAKTNTY
	CLP 2689	VATARREII
	CLP 2648	PALPGQVTI
	CLP 2680	ALPGQVTIR
	CLP 2619	RVRVPYRVV
	CLP 2681	RDRDPVFEI
	CLP 2689	RVRVPYRVV
	CLP 2947	HIAVRTGKI
	CLP 2762	NENDFLAGS
	CLP 2933	CAVRICERT
	CLP 2749	VCHITATQA
3	CLP 2647	GRREDVATA
	CLP 2677	DVATARREI
	CLP 2643	TARREIISA
	CLP 2785	GVAPALPGQ
	CLP 2917	RVVGLVVGP
	CLP 2695	VHQPGCKPL
	CLP 2650	PATSAGPEL
	CLP 2869	VTAALVPCG
4	CLP 2812	VPVPTSEHV
	CLP 2892	ARREIISAA
	CLP 2738	RIQQQTNTY
	CLP 2651	NVERAREEI
	CLP 2870	GFEAPRLDV
	CLP 2899	ATSAGPELA
	CLP 2710	FSKLGGGGL
	CLP 2599	GLRSPGGGR
5	CLP 2609	PTSEHVAEI
	CLP 2602	EIVGRQCKI
	CLP 2641	LRAKTNTYI
	CLP 2620	VTGRREDVA
	CLP 2940	SMIRASRNK
	CLP 2921	CMVCFESEV
	CLP 2936	LVPCGHNLF
	CLP 2663	NLFCMECAV
	CLP 2927	RICERTDPE
	CLP 2909	RTDPECPVC

Peptide Group	Peptide Number	Peptide Sequence
6	CLP 2766	MVTGRREDV
	CLP 2711	GLVVGPKGA
	CLP 2913	IQQQTNTYI
	CLP 2823	FLAGSPDAA
	CLP 2640	GVAETSPPL
	CLP 2698	FESEVTAAL
	CLP 2929	FCMECAVRI
7	CLP 2760	KALRAKTNT
	CLP 2633	RGEEPVFMV
	CLP 2700	SAAEHFSMI
	CLP 2835	AAFGVAPAL
	CLP 2618	VVGPKGATI
	CLP 2657	YNNENDFLA
	CLP 2674	LGCIGECGV
	CLP 2911	QENATPTSV
	CLP 2801	VCFESEVTA
8	CLP 2807	TNTYIKTPV
	CLP 2872	NKSGAAFGV
	CLP 2670	QQTNTYIIT
	CLP 2756	KILEYNNEN
	CLP 2825	CGVDSGFEA
	CLP 2843	AETSPPLWA
	CLP 2713	PLWAGQENA
	CLP 2624	VLFSSASSS
	CLP 2830	SAGPELAGL
9	CLP 2712	ISAAEHFSM
	CLP 2744	QQQTNTYII
	CLP 2774	EEIETHIAV
	CLP2819	IETHIAVRT
	CLP 2656	LAGSPDAAI
	CLP 2922	AIDSRYSDA
	CLP 2746	DVYYGVAET
	CLP 2767	VPCGHNLFC
	CLP 2950	ITATQAIRI
10	CLP 2793	TPVRGEEPV
	CLP 2777	AEHFSMIRA
	CLP 2910	VAPALPGQV
	CLP 2721	TPSRDRDPV
	CLP 2630	IAVRTGKIL
	CLP 2659	SRYSDAWRV
	CLP 2678	LSTFRQNSL
	CLP 2750	RLDVYYGVA
	CLP 2833	AGQENATPT
	CLP 2817	MVCFESEVT

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C. Immune reactivity of BCY1 peptides and generation of human effector T cells

The library of 100 peptides from BCY1 was separated into 10 groups of 7-10 peptides for immunological testing. Dissolved peptide pools were pulsed onto autologous HLA-A*0201 dendritic cells and used to activate autologous T-cell-enriched PBMC preparations. Activated T cells from each peptide-pool-stimulated culture were re-stimulated another 3 to 5 times using CD40L-activated autologous B-cells. IFN-γ ELISPOT analysis and assays for CTL killing of peptide-pulsed target cells was performed to demonstrate the immunogenicity of these epitopes from BCY1.

Human T cells demonstrated effector cell activity against a number of pools of peptides from the BCY1 protein, as shown by their ability to secrete IFN-γ in ELISPOT assays. These experiments were repeated after different rounds of APC stimulation resulting in the same reactive peptide groups. Peptide groups 1, 2, 3, 4, 5, 6, and 7 were found to be immunoreactive in these assays. Subsequently, these reactive peptide groups were de-convoluted in additional IFN-γ ELISPOT assays in which single peptides from each group were tested separately. This analysis revealed a number of individual strongly reactive peptides from the BCY1 protein recognized by human T cells (**Fig. 11**). Many of these single peptides also induced CTL activity killing peptide-loaded human T2 lymphoma cell targets. **Table IX** lists these peptides.

Example 7

BFA5/NYBR-1 Breast Cancer Antigen

A. Identification of BFA5

Microarray profiling analysis indicated that BFA5 was expressed at low to high levels in 41 out of 54 breast tumor biopsy samples (76%) and at high levels in 31 out of 54 breast tumors (57%), as compared to a panel of 52 normal, non-tumor tissues. *In situ* hybridization (ISH) was performed using a series of BFA5 DNA probes and confirmed the microarray with at least 61% of the tumors showing fairly strong signals. Further bioinformatics assessment confirmed the results of these gene expression analysis results.

Sequence analysis of the BFA5 nucleotide sequence revealed a high degree of similarity to two unidentified human genes: KIAA1074 (GenBank Accession No. XM_159732); and, KIAA0565 (GenBank Accession No. AB011137) isolated from a number of fetal and adult brain cDNA clones (Kikuno, et al. The complete sequences of 100 new cDNA clones from brain which code for large proteins *in vitro*. *DNA Res. 6: 197-205*). These genes were found to contain

putative Zn finger regions and a nuclear localization sequence. BFA5 was suggested by others to be a potential breast cancer antigen (Jager, et al. 2001. Identification of a tissue-specific putative transcription factor in breast tissue by serological screening of a breast cancer library. *Cancer Res. 61: 2055-2061* and WO 01/47959). In each of these publications, the nucleotide sequence BFA5 was designated NYBR-1 ("New York Breast Cancer-1"; GenBank Accession Nos. AF269087 (nucleotide) and AAK27325 (amino acid). For the purposes of this application, the sequence is referred to as BFA-5, the terms BFA-5 and NYBR-1 are interchangeable.

As shown previously by Jager, et al. and described in WO 01/47959, *supra*, BFA5 is specifically expressed in mammary gland, being expressed in 12/19 breast tumors analyzed. The structure of the BFA5/NYBR-1 gene has revealed that it encodes a 150-160 kD nuclear transcription factor with a bZIP site (DNA-binding domain followed by a leucine zipper motif). The gene also contains 5 tandem ankyrin repeats implying a role in protein-protein interactions. These ankyrin repeats may play a role in homo-dimerization of the protein. The BFA5 cDNA sequence is shown in **FIG. 12** and **SEQ ID NO.: 27**. The BFA5 amino acid sequence is shown in **FIG. 13** and **SEQ ID NO.: 28**.

B. Immunoreactivity of BFA5

1. Activation of human T cells and IFN-γ secretion in ELISPOT.

A library of 100 peptides from the BFA5/NYBR-1 coding sequence that are predicted to be medium to high binders to HLA-A*0201 were designed using Rammensee and Parker algorithms. The library was sub-divided into 10 pools of ten peptides (see **Table XI**), and each pool was used to activate 10 different T cell cultures after pulsing peptides on to mature autologous dendritic cells. Two experiments were performed with the library of BFA5/NYBR-1 peptides demonstrating immunoreactivity in HLA-A*0201 human T cells, as described below.

TABLE X

BFA5 Peptide Pools

Peptide Group	CLP number	Sequence		Peptide Group	CLP number	Sequence
	2983	LMDMQTFKA			3033	FESSAKIQV
	2984	KVSIPTKAL			3034	GVTAEHYAV
	2985	SIPTKALEL		BFA5	3035	RVTSNKTKV
BFA5	2986	LELKNEQTL		Group 6	3036	TVSQKDVCV
Group 1	2987	TVSQKDVCL			3037	KSQEPAFHI
	2988	SVPNKALEL			3038	KVLIAENTM
	2989	CETVSQKDV			3039	MLKLEIATL
	2990	KINGKLEES			3040	EILSVVAKL
	2991	SLVEKTPDE			3041	MLKKEIAML
	2992	SLCETVSQK			3042	LLKEKNEEI
	2993	EIDKINGKL			3043	ALRQDIEL
	2994	MLLQQNVDV			3044	KIREELGRI
BFA5	2995	NMMLQQQLV		BFA5	3045	TLKLKEESL
Group 2	2996	FLVDRKQQL		Group 7	3046	ILNEKIREE
	2997	YLLHENQML			3047	VLKKKLSEA
	2998	SLFESSAKI			3048	GTSDKIQQL
	2999	KITIDIHFL			3049	GADINLVDV
	3000	QLQSKNMML			3050	EICSVRLTL
	3001	SLDQKLFQL			3051	SVESNLNQV
	3002	FLLIKNANA			3052	SLKINLNYA
	3003	KILDTVHSC			3053	KTPDEAASL
	3004	SLSKILDTV			3054	ATQGMKVSI
	3005	ILIDSGADI		BFA5	3055	LSHGAVIEV
BFA5	3006	KVMEINREV		Group 8	3056	EIAMLKLEI
Group 3	3007	KLLSHGAVI			3057	AELQMTLKL
	3009	AVYSEILSV			3058	VFAADIQGV
	3010	KMNVDVSST			3060	PAIEMQNSV
	3011	ILSVVAKLL			3061	EIFNYNNHL
	3012	VLIAENTML			3062	ILKEKNAEL
	3013	KLSKNHQNT			3063	QLVHAHKKA
	3014	SLTPLLLSI			3065	NIQDAQKRT
BFA5	3015	SQYSGQLKV		BFA5	3066	NLVDVYGNM
Group 4	3016	KELEVKQQL		Group 9	3067	KCTALMLAV
	3017	QIMEYIRKL			3068	KIQQLEKAT
	3018	AMLKLEIAT			3069	KIAWEKKET
	3019	VLHQPLSEA			3070	IAWEKKEDT
	3020	GLLKATQGM			3071	VGMLLQQNV
	3021	GLLKANQGM			3072	VKTGCVARV
	3022	QQLEQALRI				
					3074	ALHYAVYSE
	3023	QMLKKEIAM			3075	QMKKKFCVL
	3024	EQMKKKFCV		BFA5	3076	ALQQCHQEAC
BFA5	3025	IQDIELKSV		Group 10	3077	SEQIVEFLL
Group 5	3026	SVPNKAFEL			3078	AVIEVHNKA
	3027	SIYQKVMEI			3079	AVTQGFHHI
	3028	NLNYAGDAL			3080	ACLQRKMNV
	3029	AVQDHDQIV			3081	SLVEGTSDK
	3030	LIAENTMLT				
	3031	FELKNEQTL				

ELISPOT analysis was performed on human T-cell cultures activated through four rounds of stimulation with each pool of BFA5 peptides. In **Fig. 14A**, the numbers under the X-axis indicate the number of each peptide pool (1-10). Reactivity against a CMV pp65 peptide and a Flu matrix peptide were used as positive controls for T-cell activation in the experiments. Each experiment was performed with PBMC and dendritic cells from a single HLA-A*0201^{+} donor designated as "AP10". The results show that, although BFA4 is markedly reactive with high ELISPOT counts per 100,000 cells in the assay, BFA5 is even more reactive with 9/10 pools demonstrating ELISPOT reactivity. Similar results were obtained for both BFA4 and BFA5/NYBR-1 with a different HLA-A*0201. The bars reach a maximum at 600 spots because beyond that the ELISPOT reader does not give accurate counts. Cultures having a reading of 600 spots have more than this number of spots.

A large number of the BFA5 peptide pools of are reactive as shown by the high levels of IFN-γ production (**Fig. 14A**). Each reactive peptide pool was then separated into individual peptides and analyzed for immunogencity using ELISPOT analysis to isolate single reactive BFA5 peptides. As shown in **Fig. 14B**, BFA5 is highly immunogenic with several reactive single peptides than that of BFA4. Similar results were obtained in two independent PBMC culture experiments.

In addition to ELISPOT analysis, human T cells activated by BFA5 peptides were assayed to determine their ability to function as CTL. The cells were activated using peptide-pulsed dendritic cells followed by CD40 ligand-activated B cells (5 rounds of stimulation). The experiment shown was performed with isolated PBMC from HLA-A*0201^{+} donor AP31. Isolated T cells were tested in ^{51}Cr-release assays using peptide-loaded T2 cells. The % specific lysis at a 10:1, 5:1, and 1:1 T-cell to target ratio is shown for T2 cells pulsed with either pools of BFA5/NYBR-1 peptides or with individual peptides. The graph shows CTL activity induced against targets loaded with a c non-specific HLA-A*0201-binding HIV peptide (control) followed by the CTL activity against the peptide pool (Pool 1 etc.) and then the activity induced by individual peptides from the respective pool to the right. A high level of cytotoxicity was observed for some peptides at a 1:1 E:T ratio. CTL activity (percent specific lysis) induced by the control HIV peptide was generally <10%. Similar results were obtained with another PBMC donor expressing HLA-A*0201 (AP10). **Fig. 14C** shows that a large number of BFA5 peptides trigger T cell-mediated cytotoxicity of BFA5 peptide-loaded target cells. **Table XI** lists those peptides having immunogenic properties. Five peptides (LMDMQTFKA, ILIDSGADI,

ILSVVAKLL, SQYSGQLKV, and ELCSVRLTL) were found to induce both IFN-γ secretion and CTL activity in T cells from both donors.

TABLE XI

Immunoreactive peptides from BFA5

BFA5 peptides eliciting high IFN-γ release (>200 spots/100,000 cells)		BFA5 peptides inducing CTL lysis of pulsed cells	
Donor AP10	Donor AP31	Donor AP10	Donor AP31
LMDMQTFKA	LMDMQTFKA	LMDMQTFKA	LMDMQTFKA
KVSIPTKAL			KVSIPTKAL
SIPTKALEL			SIPTKALEL
TVSQKDVCL			
SVPNKALEL			
YLLHENCML	YLLHENCML	YLLHENCML	
QLQSKNMWL	QLQSKNMWL		QLQSKNMWL
SLSKILDTV	SLSKILDTV		SLSKILDTV
ILIDSGADI	ILIDSGADI	ILIDSGADI	ILIDSGADI
KVMEINREV			
AVYSEILSV			
ILSVVAKLL	ILSVVAKLL	ILSVVAKLL	ILSVVAKLL
SLTPLLLSI	SLTPLLLSI		SLTPLLLSI
SQYSGQLKV	SQYSGQLKV	SQYSGQLKV	SQYSGQLKV
QIMEYIRKL	QIMEYIRKL		QIMEYIRKL
SVPNKAFEL			
NLNYAGDAL	NLNYAGDAL		
	GVTAEHYAV		
	KSQEPAFHI		
MLKLEIATL	MLKLEIATL		MLKLEIATL
	MLKKEIAML		
ALRIQDIEL			
	VLKKKLSEA		
ELCSVRLTL	ELCSVRLTL	ELCSVRLTL	ELCSVRLTL
SLKINLNYA	SLKINLNYA		SLKINLNYA
ATCGMKVSI		ATCGMKVSI	
AELQMTLKL		AELQMTLKL	AELQMTLKL
	VFAADICGV		
ILKEKNAEL	ILKEKNAEL		
NLVDVYGNM		NLVDVYGNM	
KCTALMLAV			

C. Immunological Reagents

Polyclonal antisera were generated against the following series of 22- to 23- mer peptides of BFA5:

BFA5(1-23)	KLH-MTKRKKTINLNIQDAQKRTALHW (CLP-2977)
BFA5(312-334)	KLH-TSEKFTWPAKGRPRKIAWEKKED (CLP-2978)
BFA5(612-634)	KLH-DEILPSESKQKDYEENSWDTESL (CLP-2979)
BFA5(972-994)	KLH-RLTLNQEEEKRRNADILNEKIRE (CLP-2980)
BFA5(1117-1139)	KLH-AENTMLTSKLKEKQDKEILEAEI (CLP-2981)
BFA5(1319-1341)	KLH-NYNNHLKNRIYQYEKEKAETENS (CLP-2982)

Prebleed samples from rabbits were processed and stored at –20°C. Rabbits were immunized as follows: 1) the peptides were administered as an emulsion with Freund's Complete Adjuvant (FCA); and, 2) two weeks later, the peptides were coupled with Keyhole-Limpet Hemocyanin (KLH)-coupled and administered as an emulsion with Freund's Incomplete Adjuvant FIA. The following results were observed:

TABLE XII

Peptide/protein	IgG titer x 10^5 (after first Immunization Rb1/Rb2)	IgG titer x 10^5 (after second Immunization Rb1/Rb2)
CLP 2977	25/6	256/64
CLP 2978	25/25	64/256
CLP 2979	12/25	256/512
CLP 2980	25/12	1024/128
CLP 2981	8/4	256/64
CLP 2982	2/2	64/32

Prebleed sample results exhibited IgG titers <100 for all samples.

To assess the quality of the polyclonal antisera, western blots were performed using sera against BFA5. Sera were separately screened against cell extracts obtained from the BT474, MDMB453, MCF-7, Calu-6, and CosA2 cells. The approximate expected MW_r of BFA5 protein is 153 kDa. A 220kD band was observed in the BT474 extract with CLP2980 antibody but not in the MDMB453 cell extracts however a ~130kD band was present in the MDMB453 extract. Both bands were found to be consistent with the polyclonal antibosera tested in this analysis. Neither of these bands is present in the negative control. Thus, it can be concluded that the polyclonal antisera are specific for BFA5.

EXAMPLE 8

BCZ4 Tumor Antigen

A. BCZ4 Sequence

The BCZ4 sequence was detected as an over-expressed sequence in breast cancer samples. The nucleotide sequence and deduced amino acid sequence of BCZ4 are shown in **Fig. 15, SEQ ID NO. 29** (BCZ4 cDNA), and **SEQ ID NO. 30** (BCZ4 amino acid sequence).

B. Immunological reagents for BCZ4 breast cancer antigen:

A library of 100 nonamer peptides spanning the BCZ4 gene product was synthesized. The peptides were chosen based on their potential ability to bind to HLA-A*0201. **Table XIII** lists 100 nonamer peptide epitopes for HLA-A*0201 from the BCZ4 protein tested (see below):

Table XIII

BCZ4 Peptide Pools

Peptide Group	CLP number	Sequence
	3220	LDLETLTDI
	3221	DILQHQIRA
	3222	ILQHQIRAV
BCZ4	3223	AVPFENLNI
Group 1	3224	NLNIHCGDA
	3225	AMDLGLEAI
	3226	GLEAIFDQV
	3227	LEAIFDQVV
	3228	WCLQVNHLL
	3229	QVNHLLYWA
	3230	VNHLLYWAL
	3231	HLLYWALTT
BCZ4	3232	LLYWALTTI
Group 2	3233	ALTTIGFET
	3234	LTTIGFETT
	3235	TTIGFETTM
	3236	TIGFETTML
	3237	TMLGGYVYS
	3238	MLGGYVYST
	3239	YSTGMIHLL
	3240	STGMIHLLL
	3241	GMIHLLLQV
	3242	MIHLLLQVT
BCZ4	3243	LLLQVTIDG
Group 3	3244	VTIDGRNYI
	3245	TIDGRNYIV
	3246	YIVDAGFGR
	3247	RSYQMWQPL
	3248	YQMWQPLEL
	3249	QMWQPLELI
	3250	ISGKDQPQV
	3251	KDQPQVPCV
BCZ4	3252	PQVPCVFRL
Group 4	3253	QVPCVFRLT
	3254	RLTEENGFW
	3255	TEENGFWYL
	3256	NGFWYLDQI
	3257	DQIRREQYI
	3258	YIPNEEFLH
	3259	YSFTLKPRT
	3260	RTIEDFESM
	3261	YLQTSPSSV
BCZ4	3262	QTSPSSVFT
Group 5	3263	SVFTSKSFC
	3264	FTSKSFCSL
	3265	CSLQTPDGV
	3266	LQTPDGVHC
	3267	QTPDGVHCL
	3268	TPDGVHCLV
	3269	GVHCLVGFT

Peptide Group	CLP number	Sequence
	3270	CLVGFTLTH
	3271	TLTHRRFNY
BCZ4	3272	FNYKDNTDL
Group 6	3273	NTDLIEFKT
	3274	TDLIEFKTL
	3275	LSEEEIEKV
	3276	KVLKNIFNI
	3277	LKNIFNISL
	3278	NISLQRKLV
	3279	KHGDRFFTI
	3280	DIEAYLERI
	3281	YLERIGYKK
BCZ4	3282	RNKLDLETL
Group 7	3283	NKLDLETLT
	3284	KLDLETLTD
	3285	DLETLTDIL
	3286	TLTDILQHQ
	3287	LTDILQHQI
	3288	QIRAVPFEN
	3289	IRAVPFENL
	3290	IHCGDAMDL
	3291	HCGDAMDLG
BCZ4	3292	DLGLEAIFD
Group 8	3293	AIFDQVVRR
	3294	GWCLQVNHL
	3295	LQVNHLLYW
	3296	GGYVYSTPA
	3297	YVYSTPAKK
	3298	STPAKKYST
	3299	IHLLLQVTI
	3300	HLLLQVTID
	3301	LLQVTIDGR
BCZ4	3302	YLDQIRREQ
Group 9	3303	QYIPNEEFL
	3304	FLHSDLLED
	3305	DLLEDSKYR
	3306	YRKIYSFTL
	3307	KIYSFTLKP
	3308	TLKPRTIED
	3309	VHCLVGFTL
	3310	LTHRRFNYK
	3311	DLIEFKTLS
BCZ4	3312	LIEFKTLSE
Group 10	3313	FKTLSEEEI
	3314	TLSEEEIEK
	3315	EIEKVLKNI
	3316	FNISLQRKL
	3317	SLQRKLVPK
	3318	KLVPKHGDR
	3319	PKHGDRFFT

C. Immune reactivity of BCZ4 peptides and generation of human effector T cells

Human PBMC from an HLA-A2.1 positive donor designated AP10 were activated with autologous dendritic cells pulsed with different pools of 9-mer peptides from the BCZ4 antigen (see **Table XIII** for list). The activated T cells were re-stimulated after 12 days with activated autologous CD40-ligand-activated B cells pulsed with the same respective peptide pools for another 8 to 10 days. This secondary activation was repeated more time for a total of 3 stimulations. The activated T cells were isolated after the 3rd stimulation and subjected to ELISPOT analysis for human IFN-γ production against their respective BCZ4 peptide pools as shown (**Fig. 16A**). In **Fig. 16A**, the blue bars show reactivity against the BCZ4 peptide pools and the red bars are for an HLA-A2.1-binding HIV peptide as a negative control. Positive control HLA-A2.1-binding recall antigen peptides for CMV and flu were as used as positive control in the experiment. Standard deviations are indicated. The experiment was repeatedon activated T cells after an additional round of peptide stimulation with the similar results.

The peptide pools were deconvoluted using IFN-γ ELISPOT assays (**Fig. 16 B**). Human T cells from donor AP10 were stimulated with the different pools of BCZ4 peptides shown in **Table XIII**. Stimulation was performed as described earlier for the other antigens described. After 4 and 5 rounds of stimulation, T cells were harvested and subjected to ELISPOT analysis for IFN-γ production with each individual peptide in each pool. The bars shown represent individual peptide reactivity for each specific pool. Table XIII identifies each of the reactive peptides. This experiment was repeated with similar results following another round of stimulation of AP10 donor T cells.

In addition to ELISPOT analysis, human T cells activated by BCZ4 peptides were assayed to determine their ability to function as CTL. The cells were activated using peptide-pulsed dendritic cells followed by CD40 ligand-activated B cells (5 rounds of stimulation). The experiment shown was performed with isolated PBMC from HLA-A*0201^{+} donor AP31. Isolated T cells were tested in ^{51}Cr-release assays using peptide-loaded T2 cells. The % specific lysis at a 10:1 T-cell to target ratio is shown for T2 cells pulsed with individual BCZ4 peptides. A high level of cytotoxicity was observed for some peptides (**Fig. 16C**). CTL activity (percent specific lysis) induced by the control HIV peptide was generally <10%. Similar results were obtained with another PBMC donor expressing HLA-A*0201 (AP10).

Table XIV lists the reactivity of the individual peptides:

TABLE XIV

Peptides eliciting strong IFN-γ ELISPOT activity		Peptides eliciting CTL activity (peptide pulsed targets)
CLP 3222	ILQHQIRAV	ILQHQIRAV
CLP 3225	AMDLGLEAI	
CLP 3226	GLEAIFDQV	GLEAIFDQV
CLP 3227	LEAIFDQVV	
CLP 3229	QVNHLLYWA	
CLP 3231	HLLYWALTT	
CLP 3232	LLYWALTTI	LLYWALTTI
CLP 3235	TTIGFETTM	
CLP 3237	TMLGGYVYS	
CLP 3239	YSTGMIHLL	
CLP 3240	STGMIHLLL	
CLP 3248	YQMWQPLEL	YQMWQPLEL
CLP 3260	RTIEDFESM	
CLP 3261	YLQTSPSSV	YLQTSPSSV
CLP 3266	LQTPDGVHC	
CLP 3267	QTPDGVHCL	
CLP 3268	TPDGVHCLV	
CLP 3269	GVHCLVGFT	
CLP 3271	TLTHRRFNY	
CLP 3277	LKNIFNISL	
CLP 3288	QIRAVPFEN	
CLP 3289	IRAVPFENL	
CLP 3294	GWCLQVNHL	
CLP 3298	STPAKKYST	
CLP 3299	IHLLLQVTI	IHLLLQVTI
CLP 3301	LLQVTIDGR	
CLP 3306	YRKIYSFTL	
CLP 3307	KIYSFTLKP	
CLP 3308	TLKPRTIED	
CLP 3309	VHCLVGFTL	
CLP 3317	SLQRKLVPK	
CLP 3319	PKHGDRFFT	

D. BCZ4 Expression Vectors

BCZ4 was PCR amplified using plasmid called pSporty/BCZ4 as the template using Platinum Taq (Invitrogen). Amplification conditions were as follows: 1) 94°C 2 minutes; 2) 35

cycles of 94°C 30 seconds, 53°C 30 seconds, 67°C 2.5 minutes; and, 3) 67°C 7 minutes. PCR primers were designed to include EcoRI restriction sites and directly flank the ORF (i.e., no extraneous sequence). Primer sequences were as follows:

AS032F (forward primer) 5' GGAATTC*AAC*ATGGACATTGAAGCATATCTTGAAAGAATTG 3' AS034R (reverse primer) 5' GGAATTCCTGGTGAGCTGGATGACAAATAGAC AAGATTG 3'. A Kozak sequence was also included in the forward primer. pcDNA3.1/Zeo(+) was cut with EcoRI and treated with CIP to prevent self-ligation. The BCZ4 amplicon was then ligated into EcoRI digested pcDNA3.1/Zeo(+). Sequencing produced one clone (AS-579-5) which matched the expected BCZ4 sequence. BCZ4 protein was then expressed from this expression vector using standard techniques.

EXAMPLE 9

BFY3 Tumor Antigen

A. BFY3 Sequence

The BFY3 sequence was detected as an over-expressed sequence in breast cancer samples. RT-PCR amplification of BFY3 w/EcoRI ends from HTB131 total RNA with AS007F (forward primer) 5' **GGAATTC*ACC*ATG**CTTTGGAAATTGACGGAT 3' and AS010R (reverse primer) 5' **GGAATTCCTCA**CTTTCTGTGCT TCTC CTCTTTGTCA 3' was performed. PCR product was digested with EcoR1 and cloned into EcoRI digested and CIP treated pcDNA3.1/Zeo(+) vector by ligation. Several positive clones were identified by restriction digestion and sequence results of AS-391-2 match expected BFY3 sequence. The nucleotide sequence and deduced amino acid sequence of BFY3 are shown in **Fig. 17, SEQ ID NO. 31** (BFY3 cDNA), and **SEQ ID NO. 32** (BFY3 amino acid sequence).

B. Immunological reagents for BFY3 breast cancer antigen

A library of 100 nonamer peptides spanning the BFY3 gene product was synthesized. The peptides were chosen based on their potential ability to bind to HLA-A*0201. **Table XV** lists 100 nonamer peptide epitopes for HLA-A*0201 from the BFY3 protein tested (see below):

Table XV: BFY3 Peptide Pools Used to Activate Human T Cells

Peptide Group	CLP number	Sequence
	3320	MLWKLTDNI
	3321	KLTDNIKYE
	3322	GTSNGTARL
BFY3	3323	NGTARLPQL
Group 1	3324	ARLPQLGTV
	3325	GTVGQSPYT
	3326	SPYTSAPPL
	3327	FQPPYFPPP
	3328	YFPPPYQPI
	3329	QSQDPYSHV
	3330	SHVNDPYSL
	3331	SLNPLHAQP
BFY3	3332	RQSQESGLL
Group 2	3333	GLLHTHRGL
	3334	GLPHQLSGL
	3335	GLDPRRDYR
	3336	DLLHGPHAL
	3337	LLHGPHALS
	3338	ALSSGLGDL
	3339	SSGLGDLSI
	3340	GLGDLSIHS
	3341	LGDLSIHSL
	3342	SIHSLPHAI
BFY3	3343	SLPHAIEEV
Group 3	3344	HAIEEVPHV
	3345	GINIPDQTV
	3346	QTVIKKGPV
	3347	VIKKGPVSL
	3348	SLSKSNSNA
	3349	SNSNAVSAI
	3350	AIPINKDNL
	3351	NLFGGVVNP
BFY3	3352	FGGVVNPNE
Group 4	3353	GGVVNPNEV
	3355	NPNEVFCSV
	3356	CSVPGRLSL
	3357	SVPGRLSLL
	3358	SLLSSTSKY
	3359	LLSSTSKYK
	3360	LSSTSKYKV
	3361	STSKYKVTV
BFY3	3362	KYKVTVAEV
Group 5	3363	YKVTVAEVQ
	3364	TVAEVQRRL
	3365	RLSPPECLN
	3366	LNASLLGGV
	3367	NASLLGGVL
	3368	SLLGGVLRR
	3369	LLGGVLRRA

Peptide Group	CLP number	Sequence
	3370	VLRRAKSKN
	3371	SLREKLDKI
BFY3	3372	KLDKIGLNL
Group 6	3373	KIGLNLPAG
	3374	GLNLPAGRR
	3375	NLPAGRRKA
	3376	AGRRKAANV
	3377	RKAANVTLL
	3378	KAANVTLLT
	3379	ANVTLLTSL
	3380	NVTLLTSLV
	3381	TLLTSLVEG
BFY3	3382	LLTSLVEGE
Group 7	3383	TSLVEGEAV
	3384	SLVEGEAVH
	3385	LVEGEAVHL
	3386	VEGEAVHLA
	3387	HLARDFGYV
	3388	YVCETEFPA
	3389	CETEFPAKA
	3390	AKAVAEFLN
	3391	AVAEFLNRQ
BFY3	3392	FLNRQHSDP
Group 8	3393	QVTRKNMLL
	3394	NMLLATKQI
	3395	MLLATKQIC
	3396	LLATKQICK
	3397	QICKEFTDL
	3398	ICKEFTDLL
	3399	LLAQDRSPL
	3400	ILEPGIQSC
	3401	LEPGIQSCL
BFY3	3402	QSCLTHFNL
Group 9	3403	SCLTHFNLI
	3404	NLISHGFGS
	3405	LISHGFGSP
	3406	ISHGFGSPA
	3407	SHGFGSPAV
	3408	FGSPAVCAA
	3409	GSPAVCAAV
	3410	AVCAAVTAL
	3411	AVTALQNYL
BFY3	3412	VTALQNYLT
Group 10	3413	ALQNYLTEA
	3414	LQNYLTEAL
	3415	YLTEALKAM
	3416	LKAMDKMYL
	3417	AMDKMYLSN
	3418	KMYLSNNPN
	3419	YLSNNPNSH

Human PBMC from an HLA-A2.1 positive donor designated AP31 were activated with autologous dendritic cells pulsed with different pools of 9-mer peptides from the BFY3 antigen (see Table 1 for list). The activated T cells were re-stimulated after 12 days with activated autologous CD40-ligand-activated B cells pulsed with the same respective peptide pools for another 8 to 10 days. This secondary activation was repeated 2 more time for a total of 4 stimulations. The activated T cells were isolated after the 4^{th} stimulation and subjected to ELISPOT analysis for human IFN-γ production against their respective BFY3 peptide pools as shown. The blue bars show reactivity against the BFY3 peptide pools and the red bars are for an HLA-A2.1-binding HIV peptide as a negative control. Standard deviations are indicated. The experiment was repeated 2 times on activated T cells from different rounds of peptide stimulation with the similar results (**Fig. 18A**).

The BFY3 peptide pools were deconvoluted and studied in IFN-γ ELISPOT assays. Human T cells from donor AP10 were stimulated with the different pools of BFY3 peptides shown in Table XV. Stimulation was performed as described earlier for the other antigens described. After 4 rounds of stimulation, the T cells from each culture were harvested and subjected to ELISPOT analysis for IFN-γ production with each individual peptide in each pool. **Fig. 18B** illustrates individual peptide reactivity for each specific pool.

In addition to ELISPOT analysis, human T cells activated by BFY3 peptides were assayed for reactivity. Ten pools of peptides consisting of ten peptides per pool used to generate CTL. These 10 groups of effectors used to kill targets pulsed with corresponding peptide pools. Peptides from pools 1, 3, 5, 6, and 7 found to be recognized, indicating that peptides in those pools are capable of generating CTL (**Fig. 18C**). From these ten pools, peptides 3344, 3320, 3378, 2272, and 3387 were strongly recongized by CTL (**Fig. 18D**). "Moderately recognized" peptides include 3369, 3355, and 3362 (**Fig. 18D**). CosA2 cells transfected with BFY3 were killed by CTL generated from pools 1 and 3 indicating that processed and presented epitopes from these pools are immunologically relevant (**Fig. 18E**). The peptides responsible for this cytotoxicity are 3320 and 3344. Table XVI summarizes the properties of the BFY3 peptides.

Table XVI

Summary of Immunoreactive BFY3 Nonamer Peptides

Peptides eliciting IFN-γ ELISPOT activity		Peptides eliciting CTL activity (peptide pulsed targets)
CLP 3320	**MLWKLTDNI**	**MLWKLTDNI**
CLP 3343	**SLPHAIEEV**	
CLP 3344	**HAIEEVPHV**	**HAIEEVPHV**
CLP 3351	**NLFGGVVNP**	
CLP 3362	**KYKVTVAEV**	**KYKVTVAEV**
CLP 3366	**LNASLLGGV**	
CLP 3369	**LLGGVLRRA**	**LLGGVLRRA**
CLP 3372	KLDKIGLNL	**KLDKIGLNL**
CLP 3378	KAANVTLLT	**KAANVTLLT**
CLP 3380	**NVTLLTSLV**	
CLP 3387	**KAANVTLLT**	**KAANVTLLT**
CLP 3403	**SCLTHFNLI**	
CLP 3407	**SHGFGSPAV**	
CLP 3415	YLTEALKAM	

C. BFY3 Expression Vectors

To construct a BFY3 expression vector, RT-PCR amplification of BFY3 w/EcoRI ends from HTB131 total RNA with AS007F (forward primer) 5' GGAATTC*ACC*ATGCTTTGGAAATTGACGGAT 3' and AS010R (reverse primer) 5' GGAATTCCTCACTTTCTGTGCTTCTCCTCTTTGTCA 3' was performed. PCR was performed using standard techniques. The amplified product was digested with EcoRI and cloned into CIP treated pcDNA3.1/Zeo(+) vector by ligation using standard techniques. Several positive clones were identified by restriction digestion and sequenced. Sequencing indicated that the sequence of clone AS-391-2 matched the expected BFY3 sequence. BFY3 protein was then expressed using from the BFY3 expression vector using standard techniques.

EXAMPLE 10

Expression Vectors Encoding Multiple Tumor Antigens

In certain instances, it may be desirable to construct expression vectors encoding multiple tumor antigens. It has been determined that certain combinations of antigens, when combined into a single expression vector, encompasses the expression profiles of many patients in a single vector. For instance, one study of breast cancer samples from different patients indicated that the combination of BFA4 and BFA5 covered expression profiles of 74% of the samples; the combination of BCY1 and BFA5 covered 65% of the samples; the combination of BCZ4 and BFA5 covered 69% of the samples; the combination of BFY3 and BFA5 covered 67% of the samples; the combination of BCY1, BFA4 and BFA5 covered 78% of the samples; the combination of BCZ4, BFA4 and BFA5 covered 81% of the samples; and, the combination of BFY3, BFA4, and BFA5 covered 74% of the samples. Accordingly, a multi-antigen expression construct may be built such that the most common expression profiles among breast cancer patients may be addressed using a single vector. Such a multiantigen expression vector is constructed using standard cloning techniques positioning nucleic acids encoding each of the tumor antigen sequences in proximity to a promoter or other transcriptional regulatory sequence. The expression vector may be engineered such that each nucleotide sequence encoding a tumor antigen is operably linked to a specific promoter, or the tumor antigens may collectively be operably linked to a single promoter and expressed as a single expression unit. Where a single expression unit is constructed, nucleotide sequences useful in separating the tumor antigen sequences following expression may be inserted between the tumor antigen sequences. Sequences useful for include IRES sequences, nucleotide sequences encoding amino acid sequences corresponding to protease cleavage sites, and the like. Suitable vectors for constructing such multiantigen expression vectors include, for example, poxviruses such as vaccinia, avipox, ALVAC and NYVAC.

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations that come within the scope of the invention as claimed.

CLAIMS

What is claimed is:

1. An expression vector comprising the nucleic acid sequence as illustrated in SEQ ID NO.: 29 or SEQ ID NO.: 31; a nucleic acid sequence encoding the amino acid sequence illustrated in SEQ ID NO.: 30 or SEQ ID NO.: 32; or a fragment thereof.
2. The expression vector of claim 1 wherein the vector is a plasmid or a viral vector.
3. The expression vector of claim 2 wherein the viral vector is selected from the group consisting of poxvirus, adenovirus, retrovirus, herpesvirus, and adeno-associated virus.
4. The expression vector of claim 3 wherein the viral vector is a poxvirus selected from the group consisting of vaccinia, NYVAC, avipox, canarypox, ALVAC, ALVAC(2), fowlpox, and TROVAC.
5. The expression vector of claim 4 wherein the viral vector is a poxvirus selected from the group consisting of NYVAC, ALVAC, and ALVAC(2).
6. The expression vector of claim 1 further comprising at least one additional tumor-associated antigen.
7. The expression vector of claim 6 wherein the vector is a plasmid or a viral vector.
8. The expression vector of claim 7 wherein the viral vector is selected from the group consisting of poxvirus, adenovirus, retrovirus, herpesvirus, and adeno-associated virus.
9. The expression vector of claim 8 wherein the viral vector is a poxvirus selected from the group consisting of vaccinia, NYVAC, avipox, canarypox, ALVAC, ALVAC(2), fowlpox, and TROVAC.
10. The expression vector of claim 9 wherein the viral vector is a poxvirus selected from the group consisting of NYVAC, ALVAC, and ALVAC(2).
11. The expression vector of claim 1 further comprising at least one nucleic sequence encoding an angiogenesis-associated antigen.
12. The expression vector of claim 11 wherein the vector is a plasmid or a viral vector.
13. The expression vector of claim 12 wherein the viral vector is selected from the group consisting of poxvirus, adenovirus, retrovirus, herpesvirus, and adeno-associated virus.
14. The expression vector of claim 13 wherein the viral vector is a poxvirus selected from the group consisting of vaccinia, NYVAC, avipox, canarypox, ALVAC, ALVAC(2), fowlpox, and TROVAC.
15. The expression vector of claim 14 wherein the viral vector is a poxvirus selected from the group consisting of NYVAC, ALVAC, and ALVAC(2).

16. The expression vector of claim 6 further comprising at least one nucleic sequence encoding an angiogenesis-associated antigen.
17. The expression vector of claim 16 wherein the vector is a plasmid or a viral vector.
18. The expression vector of claim 17 wherein the viral vector is selected from the group consisting of poxvirus, adenovirus, retrovirus, herpesvirus, and adeno-associated virus.
19. The expression vector of claim 17 wherein the viral vector is a poxvirus selected from the group consisting of vaccinia, NYVAC, avipox, canarypox, ALVAC, ALVAC(2), fowlpox, and TROVAC.
20. The poxvirus of claim 18 wherein the viral vector is a poxvirus selected from the group consisting of NYVAC, ALVAC, and ALVAC(2).
21. The expression vector of claim 1, 6, 11 or 16 further comprising at least one nucleic acid sequence encoding a co-stimulatory component.
22. The expression vector of claim 22 wherein the vector is a plasmid or a viral vector.
23. The expression vector of claim 23 wherein the viral vector is selected from the group consisting of poxvirus, adenovirus, retrovirus, herpesvirus, and adeno-associated virus.
24. The expression vector of claim 24 wherein the viral vector is a poxvirus selected from the group consisting of vaccinia, NYVAC, avipox, canarypox, ALVAC, ALVAC(2), fowlpox, and TROVAC.
25. The poxvirus of claim 18 wherein the viral vector is a poxvirus selected from the group consisting of NYVAC, ALVAC, and ALVAC(2).
26. A composition comprising an expression vector in a pharmaceutically acceptable carrier, said vector comprising the nucleic acid sequence shown in SEQ ID NO.: 29 or SEQ ID NO.: 31; a nucleic acid sequence encoding the amino acid sequence illustrated in SEQ ID NO.: 30 or SEQ ID NO.: 32; or a fragment thereof.
27. The expression vector of claim 26 wherein the vector is a plasmid or a viral vector.
28. The expression vector of claim 27 wherein the viral vector is selected from the group consisting of poxvirus, adenovirus, retrovirus, herpesvirus, and adeno-associated virus.
29. The expression vector of claim 28 wherein the viral vector is a poxvirus selected from the group consisting of vaccinia, NYVAC, avipox, canarypox, ALVAC, ALVAC(2), fowlpox, and TROVAC.
30. The poxvirus of claim 29 wherein the viral vector is a poxvirus selected from the group consisting of NYVAC, ALVAC, and ALVAC(2).

31. A method for preventing or treating cancer comprising administering to a host an expression vector comprising the nucleic acid sequence illustrated in SEQ ID NO.: 29 or SEQ ID NO.: 31; a nucleic acid sequence encoding the amino acid sequence illustrated in SEQ ID NO.: 30 or SEQ ID NO.: 32; or a fragment thereof.
32. The expression vector of claim 31 wherein the vector is a plasmid or a viral vector.
33. The expression vector of claim 32 wherein the viral vector is selected from the group consisting of poxvirus, adenovirus, retrovirus, herpesvirus, and adeno-associated virus.
34. The expression vector of claim 33 wherein the viral vector is a poxvirus selected from the group consisting of vaccinia, NYVAC, avipox, canarypox, ALVAC, ALVAC(2), fowlpox, and TROVAC.
35. The poxvirus of claim 34 wherein the viral vector is a poxvirus selected from the group consisting of NYVAC, ALVAC, and ALVAC(2).
36. An isolated peptide derived from BFY3 as shown in Table XV or XVI.
37. A method for immunizing a host against the tumor antigen BFY3 comprising administering to the patient a peptide shown in Table XV or XVI, either alone or in combination with another agent, where the individual components of the combination are administered simultaneously or separately from one another.
38. An isolated peptide derived from BFY3 as shown in Table XV or XVI.
39. A method for immunizing a host against the tumor antigen BFY3 comprising administering to the patient a peptide shown in Table XV or XVI, either alone or in combination with another agent, where the individual components of the combination are administered simultaneously or separately from one another.
40. An isolated peptide derived from BCZ4 as shown in Table XIII or XVI.
41. A method for immunizing a host against the tumor antigen BCZ4 comprising administering to the patient a peptide shown in Table XIII or XIV, either alone or in combination with another agent, where the individual components of the combination are administered simultaneously or separately from one another.
42. An isolated peptide derived from BCZ4 as shown in Table XIII or XVI.
43. A method for immunizing a host against the tumor antigen BCZ4 comprising administering to the patient a peptide shown in Table XIII or XVI, either alone or in combination with another agent, where the individual components of the combination are administered simultaneously or separately from one another.

44. A expression vector for expression of multiple tumor antigens or fragments thereof, the expression vector comprising at least two nucleic acid sequences encoding at least two different tumor antigens or fragments thereof, the tumor antigens being selected from the group consisting of BFA4, BCY1, BFA5, BCZ4, and BFY3.

45. A expression vector for expression of multiple tumor antigens or fragments thereof, the expression vector comprising at least two nucleic acid sequences encoding at least two different tumor antigens or fragments thereof, the nucleic acid sequences being selected from the group consisting of SEQ ID NO.: 23, SEQ ID NO.: 25, SEQ ID NO.: 27, SEQ ID NO.: 29, and SEQ ID NO.: 31.

46. The expression vector of claim 44 or 45 wherein the vector is a plasmid or a viral vector.

47. The expression vector of claim 46 wherein the viral vector is selected from the group consisting of poxvirus, adenovirus, retrovirus, herpesvirus, and adeno-associated virus.

48. The expression vector of claim 47 wherein the viral vector is a poxvirus selected from the group consisting of vaccinia, NYVAC, avipox, canarypox, ALVAC, ALVAC(2), fowlpox, and TROVAC.

49. The expression vector of claim 48 wherein the viral vector is a poxvirus selected from the group consisting of NYVAC, ALVAC, and ALVAC(2).

50. The expression vector of any one of claims 44 to 49 further comprising at least one nucleic acid sequence encoding a co-stimulatory component.

FIGURE 1A

```
AAC2-1  ATGGGTTCCCCCGCCGCCCCGGAGGGAGCGCTGGGCTACGTCCGCGAGTTCACTCGCCACTCCT
AAC2-2  ATGGGTTCCCCCGCCGCCCCGGAGGGAGCGCTGGGCTACGTCCGCGAGTTCACTCGCCACTCCT

AAC2-1  CCGACGTGCTGGGCAACCTCAACGAGCTGCGCCTGCGCGGGATCCTCACTGACGTCACGCTGCT
AAC2-2  CCGACGTGCTGGGCAACCTCAACGAGCTGCGCCTGCGCGGGATCCTCACTGACGTCACGCTGCT

AAC2-1  GGTTGGCGGGCAACCCCTCAGAGCACACAAGGCAGTTCTCATCGCCTGCAGTGGCTTCTTCTAT
AAC2-2  GGTTGGCGGGCAACCCCTCAGAGCACACAAGGCAGTTCTCATCGCCTGCAGTGGCTTCTTCTAT

AAC2-1  TCAATTTTCCGGGGCCGTGCGGGAGTCGGGGTGGACGTGCTCTCTCTGCCCGGGGGTCCCGAAG
AAC2-2  TCAATTTTCCGGGGCCGTGCGGGAGTCGGGGTGGACGTGCTCTCTCTGCCCGGGGGTCCCGAAG

AAC2-1  CGAGAGGCTTCGCCCCTCTATTGGACTTCATGTACACTTCGCGCCTGCGCCTCTCTCCAGCCAC
AAC2-2  CGAGAGGCTTCGCCCCTCTATTGGACTTCATGTACACTTCGCGCCTGCGCCTCTCTCCAGCCAC

AAC2-1  TGCACCAGCAGTCCTAGCGGCCGCCACCTATTTGCAGATGGAGCACGTGGTCCAGGCATGCCAC
AAC2-2  TGCACCAGCAGTCCTAGCGGCCGCCACCTATTTGCAGATGGAGCACGTGGTCCAGGCATGCCAC

AAC2-1  CGCTTCATCCAGGCCAGCTATGAACCTCTGGGCATCTCCCTGCGCCCCCTGGAAGCAGAACCCC
AAC2-2  CGCTTCATCCAGGCCAGCTATGAACCTCTGGGCATCTCCCTGCGCCCCCTGGAAGCAGAACCCC

AAC2-1  CAACACCCCCAACGGCCCCTCCACCAGGTAGTCCCAGGCGCTCCGAAGGACACCCAGACCCACC
AAC2-2  CAACACCCCCAACGGCCCCTCCACCAGGTAGTCCCAGGCGCTCCGAAGGACACCCAGACCCACC

AAC2-1  TACTGAATCTCGAAGCTGCAGTCAAGGCCCCCCCAGTCCAGCCAGCCCTGACCCCAAGGCCTGC
AAC2-2  TACTGAATCTCGAAGCTGCAGTCAAGGCCCCCCCAGTCCAGCCAGCCCTGACCCCAAGGCCTGC

AAC2-1  AACTGGAAAAAGTACAAGTACATCGTGCTAAACTCTCAGGCCTCCCAAGCAGGGAGCCTGGTCG
AAC2-2  AACTGGAAAAAGTACAAGTACATCGTGCTAAACTCTCAGGCCTCCCAAGCAGGGAGCCTGGTCG

AAC2-1  GGGAGAGAAGTTCTGGTCAACCTTGCCCCCAAGCCAGGCTCCCCAGTGGAGACGAGGCCTCCAG
AAC2-2  GGGAGAGAAGTTCTGGTCAACCTTGCCCCCAAGCCAGGCTCCCCAGTGGAGACGAGGCCTCCAG

AAC2-1  CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGTGAAGAAGGACCCATTCCTGGTCCCCAGAGCAGG
AAC2-2  CAGCAGCAGCAGCAGCAGCAGCAGC***AGTGAAGAAGGACCCATTCCTGGTCCCCAGAGCAGG

AAC2-1  CTCTCTCCAACTGCTGCCACTGTGCAGTTCAAATGTGGGGCTCCAGCCAGTACCCCCTACCTCC
AAC2-2  CTCTCTCCAACTGCTGCCACTGTGCAGTTCAAATGTGGGGCTCCAGCCAGTACCCCCTACCTCC

AAC2-1  TCACATCCCAGGCTCAAGACACCTCTGGATCACCCTCTGAACGGGCTCGTCCACTACCGGGA*G
AAC2-2  TCACATCCCAGGCTCAAGACACCTCTGGATCACCCTCTGAACGGGCTCGTCCACTACCGGGAAG

AAC2-1  TGAATTTTTCAGCTGCCAGAACTGTGAGGCTGTGGCAGGGTGCTCATCGGGGGCTGGACTCCTT
AAC2-2  TGAATTTTTCAGCTGCCAGAACTGTGAGGCTGTGGCAGGGTGCTCATCGGGG*CTGGACTCCTT

AAC2-1  GGTTCCTGGGGACGAAGACAAACCCTATAAGTGTCAGCTGTGCCGGTCTTCGTTCCGCTACAAG
AAC2-2  GGTTCCTGGGGACGAAGACAAACCCTATAAGTGTCAGCTGTGCCGGTCTTCGTTCCGCTACAAG

AAC2-1  GGCAACCTTGCCAGTCACCGTACAGTGCACACAGGGGAAAAGCCTTACCACTGCTCAATCTGCG
AAC2-2  GGCAACCTTGCCAGTCATCGTACAGTGCACACAGGGGAAAAGCCTTACCACTGCTCAATCTGCG
```

FIGURE 1A

```
AAC2-1   GAGCCCGTTTTAACCGGCCAGCAAACCTGAAAACGCACAGCCGCATCCATTCGGGAGAGAAGCC
AAC2-2   GAGCCCGTTTTAACCGGCCAGCAAACCTGAAAACGCACAGCCGCATCCATTCGGGAGAGAAGCC

AAC2-1   GTATAAGTGTGAGACGTGCGGCTCGCGCTTTGTACAGGTGGCACATCTGCGGGCGCACGTGCTG
AAC2-2   GTATAAGTGTGAGACGTGCGGCTCGCGCTTTGTACAGGTGGCACATCTGCGGGCGCACGTGCTG

AAC2-1   ATCCACACCGGGGAGAAGCCCTACCCTTGCCCTACCTGCGGAACCCGCTTCCGCCACCTGCAGA
AAC2-2   ATCCACACCGGGGAGAAGCCCTACCCTTGCCCTACCTGCGGAACCCGCTTCCGCCACCTGCAGA

AAC2-1   CCCTCAAGAGCCACGTTCGCATCCACACCGGAGAGAAGCCTTACCACTGCGACCCCTGTGGCCT
AAC2-2   CCCTCAAGAGCCACGTTCGCATCCACACCGGAGAGAAGCCTTACCACTGCGACCCCTGTGGCCT

AAC2-1   GCATTTCCGGCACAAGAGTCAACTGCGGCTGCATCTGCGCCAGAAACACGGAGCTGCTACCAAC
AAC2-2   GCATTTCCGGCACAAGAGTCAACTGCGGCTGCATCTGCGCCAGAAACACGGAGCTGCTACCAAC

AAC2-1   ACCAAAGTGCACTACCACATTCTCGGGGGGCCCTAG
AAC2-2   ACCAAAGTGCACTACCACATTCTCGGGGGGCCCTAG
```

FIGURE 1B

```
AAC2-1   MGSPAAPEGALGYVREFTRHSSDVLGNLNELRLRGILTDVTLLVGGQPLRAHKAVLIACSGFFYSIFRG
AAC2-2   MGSPAAPEGALGYVREFTRHSSDVLGNLNELRLRGILTDVTLLVGGQPLRAHKAVLIACSGFFYSIFRG

AAC2-1   RAGVGVDVLSLPGGPEARGFAPLLDFMYTSRLRLSPATAPAVLAAATYLQMEHVVQACHRFIQASYEPL
AAC2-2   RAGVGVDVLSLPGGPEARGFAPLLDFMYTSRLRLSPATAPAVLAAATYLQMEHVVQACHRFIQASYEPL

AAC2-1   LPGGPEARGFAPLLDFMYTSRLRLSPATAPAVLAAATYLQMEHVVQACHRFIQASYEPLGISLRPLEAE
AAC2-2   LPGGPEARGFAPLLDFMYTSRLRLSPATAPAVLAAATYLQMEHVVQACHRFIQASYEPLGISLRPLEAE

AAC2-1   PPTPPTAPPPGSPRRSEGHPDPPTESRSCSQGPPSPASPDPKACNWKKYKYIVLNSQASQAGSLVGERS
AAC2-2   PPTPPTAPPPGSPRRSEGHPDPPTESRSCSQGPPSPASPDPKACNWKKYKYIVLNSQASQAGSLVGERS

AAC2-1   SGQPCPQARLPSGDEASSSSSSSSSSSEEGPIPGPQSRLSPTAATVQFKCGAPASTPYLLTSQAQDTS
AAC2-2   SGQPCPQARLPSGDEASSSSSSSSSSS*EEGPIPGPQSRLSPTAATVQFKCGAPASTPYLLTSQAQDTS

AAC2-1   GSPSERARPLPGVNFSAARTVRLWQGAHRGLDSLVPGDEDKPYKCQLCRSSFRYKGNLASHRTVHTGEK
AAC2-2   GSPSERARPLPGSEFFSCQNCEAVAGCSSGLDSLVPGDEDKPYKCQLCRSSFRYKGNLASHRTVHTGEK

AAC2-1   PYHCSICGARFNRPANLKTHSRIHSGEKPYKCETCGSRFVQVAHLRAHVLIHTGEKPYPCPTCGTRFRH
AAC2-2   PYHCSICGARFNRPANLKTHSRIHSGEKPYKCETCGSRFVQVAHLRAHVLIHTGEKPYPCPTCGTRFRH

AAC2-1   LQTLKSHVRIHTGEKPYHCDPCGLHFRHKSQLRLHLRQKHGAATNTKVHYHILGGP
AAC2-2   LQTLKSHVRIHTGEKPYHCDPCGLHFRHKSQLRLHLRQKHGAATNTKVHYHILGGP
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FIGURE 2

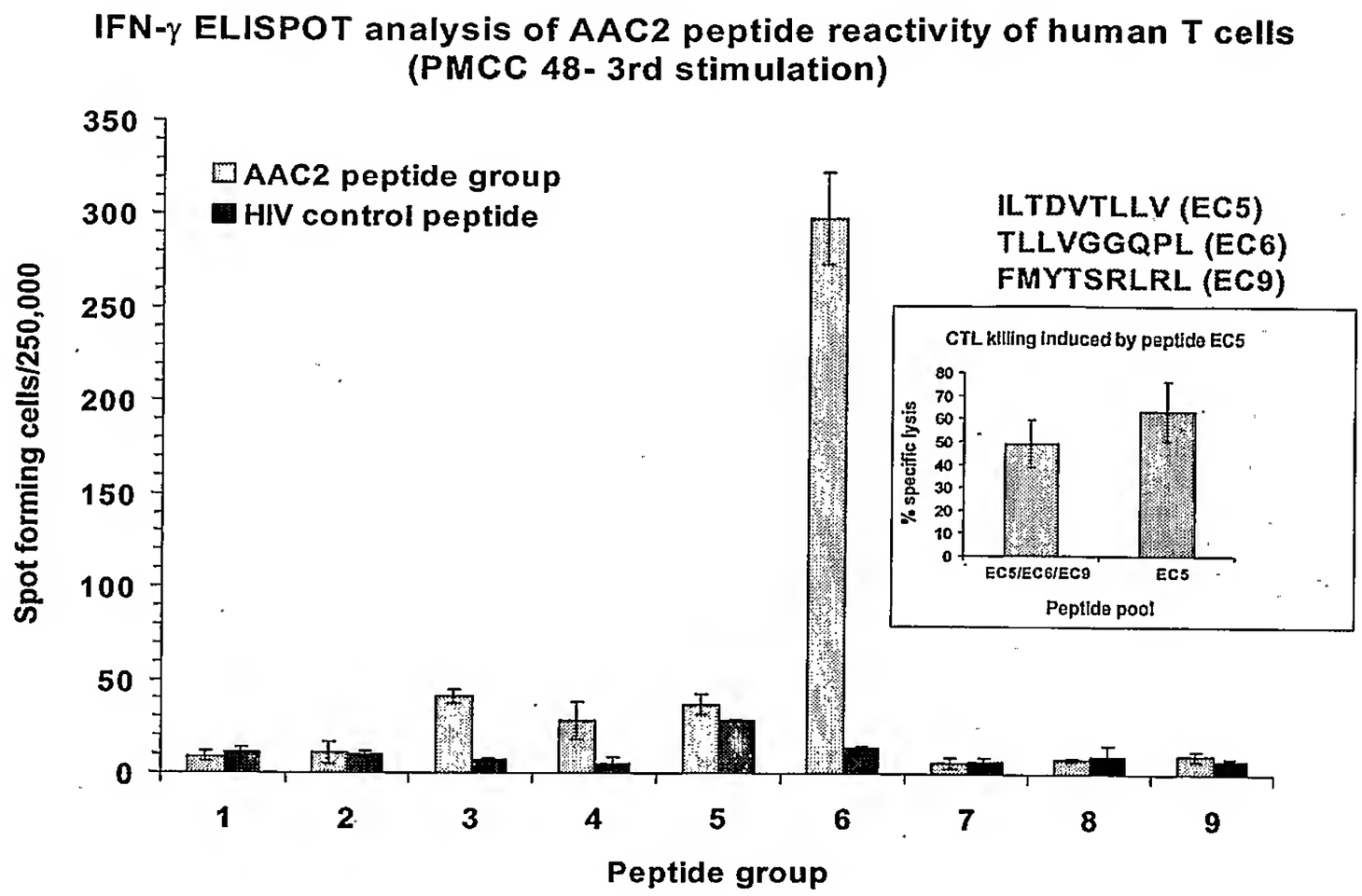
IFN-γ ELISPOT analysis of AAC2 peptide reactivity of human T cells
(PMCC 48- 3rd stimulation)
AAC2 peptide group
HIV control peptide
Spot forming cells/250,000
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
Peptide group
ILTDVTLLV (EC5)
TLLVGGQPL (EC6)
FMYTSRLRL (EC9)
CTL killing induced by peptide EC5
% specific lysis
80
70
60
50
40
30
20
10
0
EC5/EC6/EC9
EC5
Peptide pool

FIGURE 3

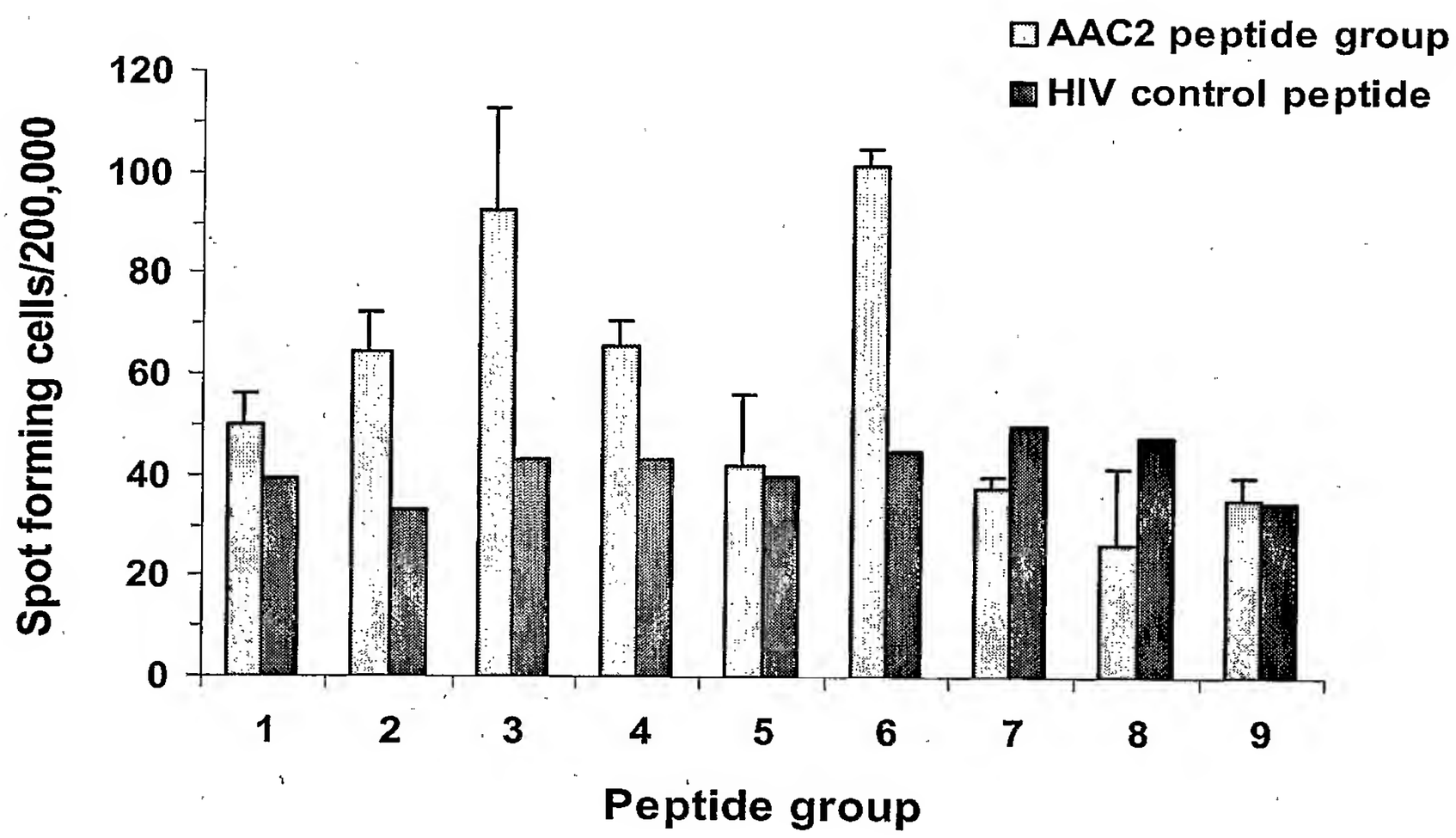
AAC2 peptide group
HIV control peptide
Spot forming cells/200,000
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
Peptide group

FIGURE 4

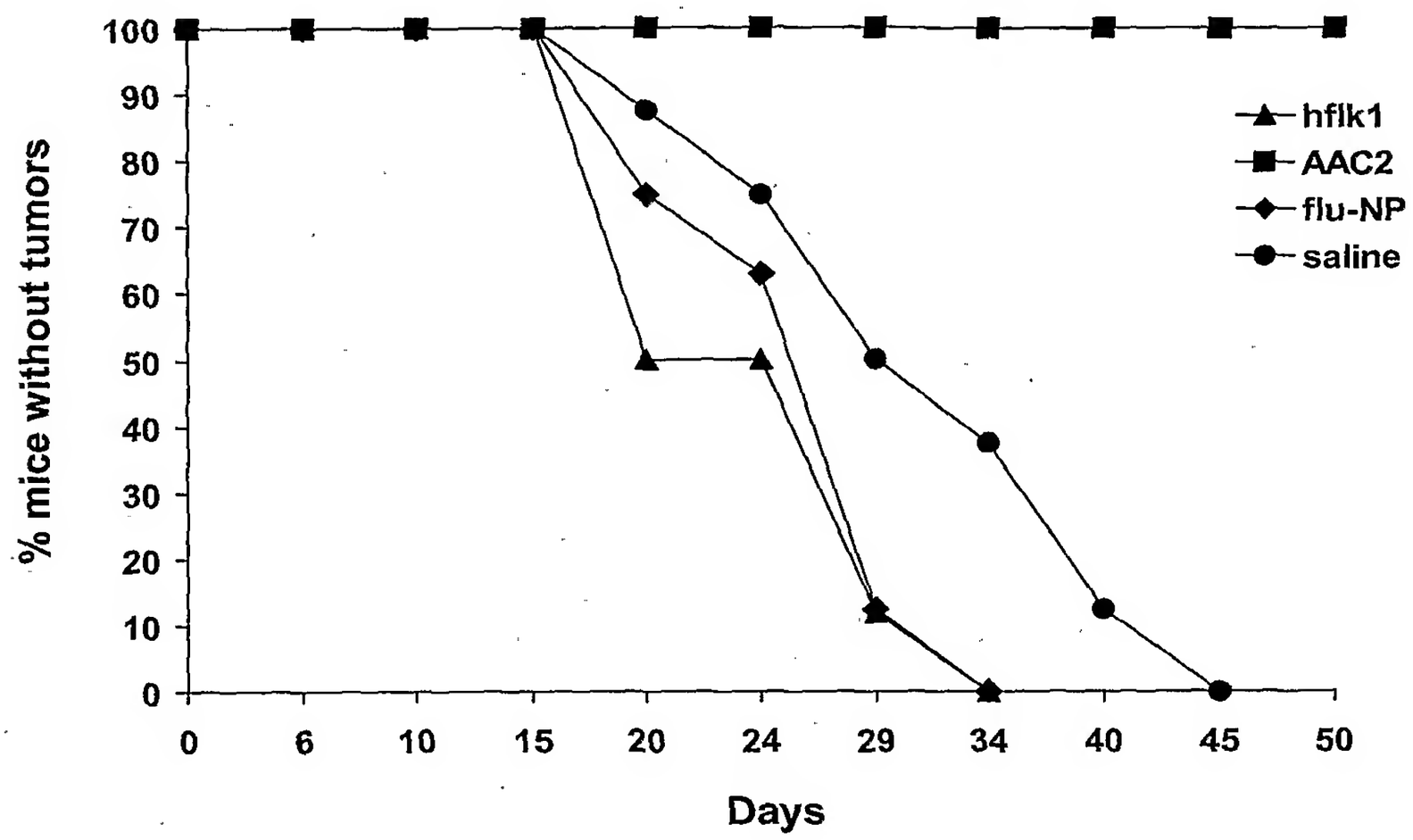
Inhibition of B16F10 melanoma growth by AAC2 vaccination
% mice without tumors
100
90
80
70
60
50
40
30
20
10
0
0
6
10
15
20
24
29
34
40
45
50
Days
hflk1
AAC2
flu-NP
saline

FIGURE 5

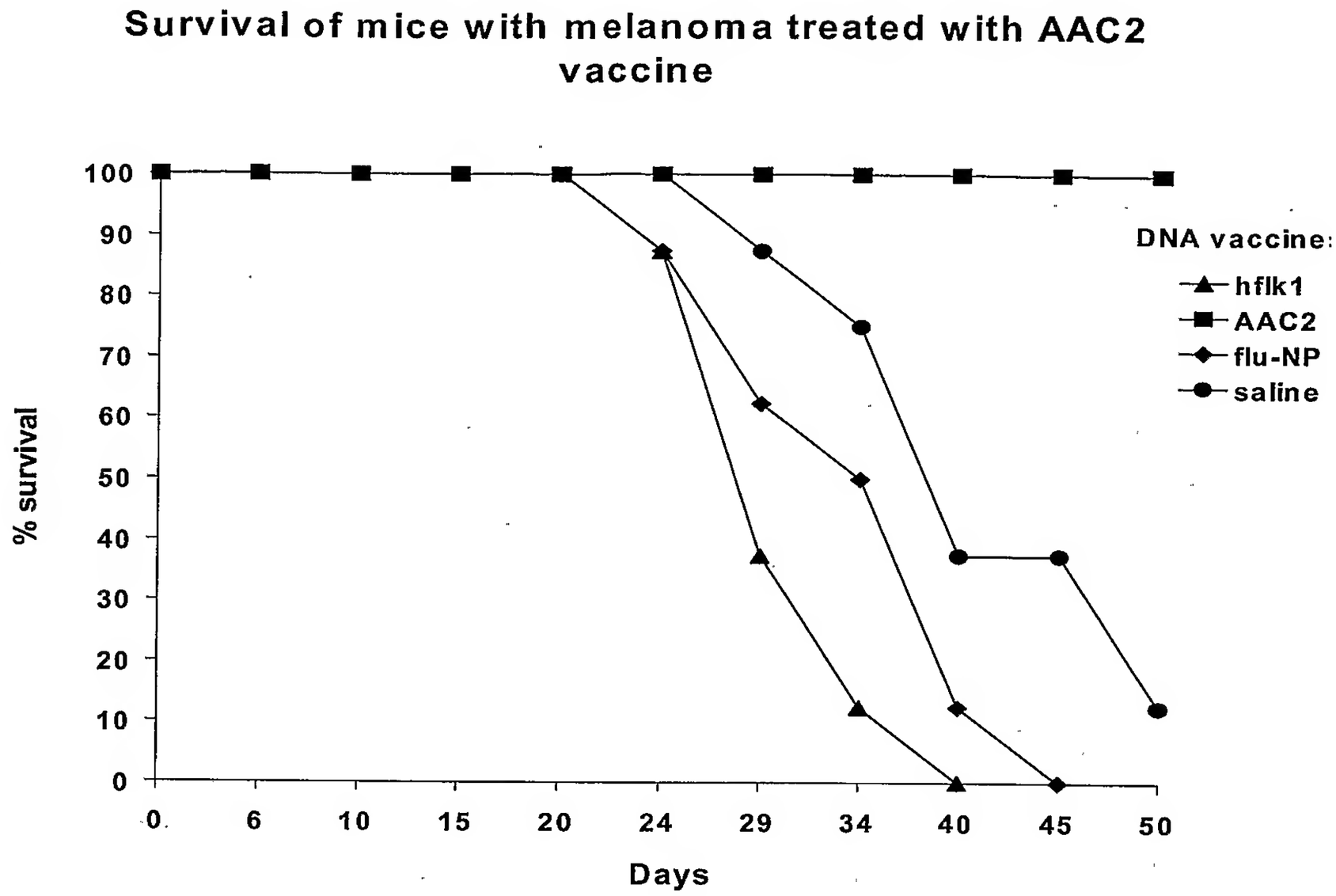
Survival of mice with melanoma treated with AAC2 vaccine
% survival
100
90
80
70
60
50
40
30
20
10
0
0
6
10
15
20
24
29
34
40
45
50
Days
DNA vaccine:
hflk1
AAC2
flu-NP
saline

FIGURE 6

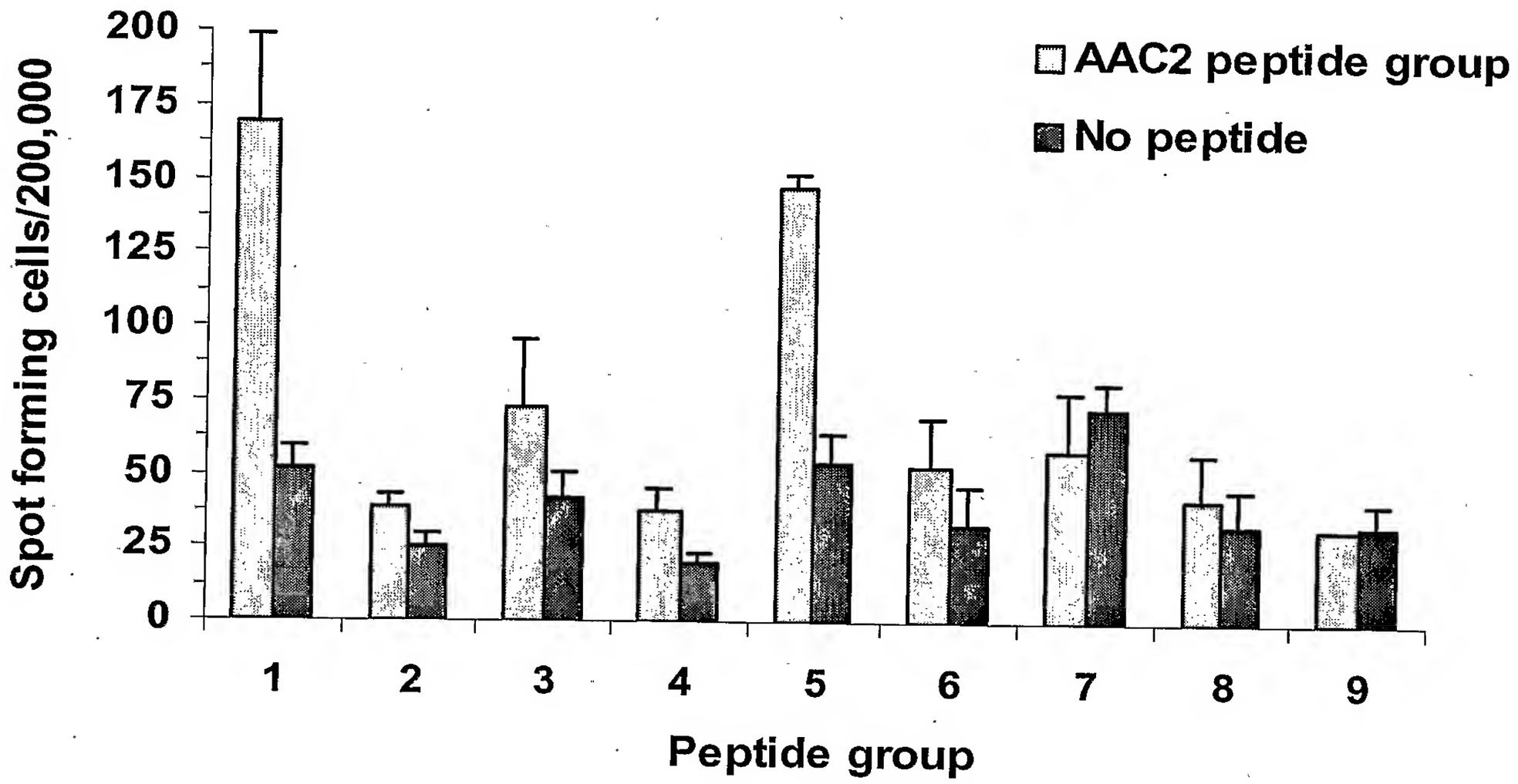
AAC2 peptide group
No peptide
Spot forming cells/200,000
200
175
150
125
100
75
50
25
0
1
2
3
4
5
6
7
8
9
Peptide group

FIGURE 7

BFA4 cDNA Sequence

ATGGTCCGGAAAAAGAACCCCCCTCTGAGAAACGTTGCAAGTGAAGGCGAGGGCCAGATCCTGGAGCCTATAGGTACAGAAAGCAA
GGTATCTGGAAAGAACAAAGAATTCTCTGCAGATCAGATGTCAGAAAATACGGATCAGAGTGATGCTGCAGAACTAAATCATAAGGA
GGAACATAGCTTGCATGTTCAAGATCCATCTTCTAGCAGTAAGAAGGACTTGAAAAGCGCAGTTCTGAGTGAGAAGGCTGGCTTCAA
TTATGAAAGCCCCAGTAAGGGAGGAAACTTTCCCTCCTTTCCGCATGATGAGGTGACAGACAGAAATATGTTGGCTTTCTCATTTCC
AGCTGCTGGGGGGAGTCTGTGAGCCCTTGAAGTCTCCGCAAAGAGCAGAGGCAGATGACCCTCAAGATATGGCCTGCACCCCCTCAGG
GGACTCACTGGAGACAAAGGAAGATCAGAAGATGTCACCAAAGGCTACAGAGGAAACAGGGCAAGCACAGAGTGGTCAAGCCAATTG
TCAAGGTTTGAGCCCAGTTTCAGTGGCCTCAAAAAACCCACAAGTGCCTTCAGATGGGGGGTGTAAGACTGAATAAATCCAAAACTGA
CTTACTGGTGAATGACAACCCAGACCCGGCACCTCTGTCTCCAGAGCTTCAGGACTTTAAATGCAATATCTGTGGATATGGTTACTA
CGGCAACGACCCCACAGATCTGATTAAGCACTTCCGAAAGTATCACTTAGGACTGCATAACCGCACCAGGCAAGATGCTGAGCTGGA
CAGCAAAATCTTGGCCCTTCATAACATGGTGCAGTTCAGCCATTCCAAAGACTTCCAGAAGGTCAACCGTTCTGTGTTTTCTGGTGT
GCTGCAGGACATCAATTCTTCAAGGCCTGTTTTACTAAATGGGACCTATGATGTGCAGGTGACTTCAGGTGGAACATTCATTGGCAT
TGGACGGAAAACACCAGATTGCCAAGGGAACACCAAGTATTTCCGCTGTAAATTCTGCAATTTCACTTATATGGGCAACTCATCCAC
CGAATTAGAACAACATTTTCTTCAGACTCACCCAAACAAAATAAAAGCTTCTCTCCCCTCCTCTGAGGTTGCAAAACCTTCAGAGAA
AAACTCTAACAAGTCCATCCCTGCACTTCAATCCAGTGATTCTGGAGACTTGGGAAAATGGCAGGACAAGATAACAGTCAAAGCAGG
AGATGACACTCCTGTTGGGTACTCAGTGCCCATAAAGCCCCTCGATTCCTCTAGACAAAATGGTACAGAGGCCACCAGTTACTACTG
GTGTAAATTTTGTAGTTTCAGCTGTGAGTCATCTAGCTCACTTAAACTGCTAGAACATTATGGCAAGCAGCACGGAGCAGTGCAGTC
AGGCGGCCTTAATCCAGAGTTAAATGATAAGCTTTCCAGGGGCTCTGTCATTAATCAGAATGATCTAGCCAAAAGTTCAGAAGGAGA
GACAATGACCAAGACAGACAAGAGCTCGAGTGGGGCTAAAAAGAAGGACTTCTCCAGCAAGGGAGCCGAGGATAATATGGTAACGAG
CTATAATTGTCAGTTCTGTGACTTCCGATATTCCAAAAGCCATGGCCCTGATGTAATTGTAGTGGGGCCACTTCTCCGTCATTATCA
ACAGCTCCATAACATTCACAAGTGTACCATTAAACACTGTCCATTCTGTCCCAGAGGACTTTGCAGCCCAGAAAAGCACCTTGGAGA
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CTCGCGAGTCAAACATCAGTGCCATCAGTGTTCATTCACCACCCCTGACGTAGATGTACTCCTCTTTCACTATGAAAGTGTGCATGA
GTCCCAAGCATCGGATGTCAAACAAGAAGCAAATCACCTGCAAGGATCGGATGGGCAGCAGTCTGTCAAGGAAAGCAAAGAACACTC
ATGTACCAAATGTGATTTTATTACCCAAGTGGAAGAAGAGATTTCCCGACACTACAGGAGAGCACACAGCTGCTACAAATGCCGTCA
GTGCAGTTTTACAGCTGCCGATACTCAGTCACTACTGGAGCACTTCAACACTGTTCACTGCCAGGAACAGGACATCACTACAGCCAA
CGGCGAAGAGGACGGTCATGCCATATCCACCATCAAAGAGGAGCCCAAAATTGACTTCAGGGTCTACAATCTGCTAACTCCAGACTC
TAAAATGGGAGAGCCAGTTTCTGAGAGTGTGGTGAAGAGAGAAGCTGGAAGAGAAGGACGGGCTCAAAGAGAAAGTTTGGACCGA
GAGTTCCAGTGATGACCTTCGCAATGTGACTTGGAGAGGGGCAGACATCCTGCGGGGGAGTCCGTCATACACCCAAGCAAGCCTGGG
GCTGCTGACGCCTGTGTCTGGCACCCAAGAGCAGACAAAGACTCTAAGGGATAGTCCCAATGTGGAGGCCGCCCATCTGGCGCGACC
TATTTATGGCTTGGCTGTGGAAACCAAGGGATTCCTGCAGGGGGCGCCAGCTGGCGGAGAGAAGTCTGGGGCCCTCCCCCAGCAGTA
TCCTGCATCGGGAGAAAACAAGTCCAAGGATGAATCCCAGTCCCTGTTACGGAGGCGTAGAGGCTCCGGTGTTTTTTGTGCCAATTG
CCTGACCACAAAGACCTCTCTCTGGCGAAAGAATGCAAATGGCGGATATGTATGCAACGCGTGTGGCCTCTACCAGAAGCTTCACTC
GACTCCCAGGCCTTTAAACATCATTAAACAAAACAACGGTGAGCAGATTATTAGGAGGAGAACAAGAAAGCGCCTTAACCCAGAGGC
ACTTCAGGCTGAGCAGCTCAACAAACAGCAGAGGGGCAGCAATGAGGAGCAAGTCAATGGAAGCCCGTTAGAGAGGAGGTCAGAAGA
TCATCTAACTGAAAGTCACCAGAGAGAAATTCCACTCCCCAGCCTAAGTAAATACGAAGCCCAGGGTTCATTGACTAAAAGCCATTC
TGCTCAGCAGCCAGTCCTGGTCAGCCAAACTCTGGATATTCACAAAAGGATGCAACCTTTGCACATTCAGATAAAAAGTCCTCAGGA
AAGTACTGGAGATCCAGGAAATAGTTCATCCGTATCTGAAGGGAAAGGAAGTTCTGAGAGAGGCAGTCCTATAGAAAAGTACATGAG
ACCTGCGAAACACCCAAATTATTCACCACCAGGCAGCCCTATTGAAAAGTACCAGTACCCACTTTTTGGACTTCCCTTTGTACATAA
TGACTTCCAGAGTGAAGCTGATTGGCTGCGGTTCTGGAGTAAATATAAGCTCTCCGTTCCTGGGAATCCGCACTACTTGAGTCACGT
GCCTGGCCTACCAAATCCTTGCCAAAACTATGTGCCTTATCCCACCTTCAATCTGCCTCCTCATTTTTCAGCTGTTGGATCAGACAA
TGACATTCCTCTAGATTTGGCGATCAAGCATTCCAGACCTGGGCCAACTGCAAACGGTGCCTCCAAGGAGAAAACGAAGGCACCACC
AAATGTAAAAAATGAAGGTCCCTTGAATGTAGTAAAAACAGAGAAAGTTGATAGAAGTACTCAAGATGAACTTTCAACAAAATGTGT
GCACTGTGGCATTGTCTTTCTGGATGAAGTGATGTATGCTTTGCATATGAGTTGCCATGGTGACAGTGGACCTTTCCAGTGCAGCAT
ATGCCAGCATCTTTGCACGGACAAATATGACTTCACAACACATATCCAGAGGGGCCTGCATAGGAACAATGCACAAGTGGAAAAAAA
TGGAAAACCTAAAGAGTAA*

FIGURE 8

BFA4 Amino Acid Sequence

MVRKKNPPLRNVASEGEGQILEPIGTESKVSGKNKEFSADQMSENTDQSDAAELNHKEEHSLHVQDPSSS
SKKDLKSAVLSEKAGFNYESPSKGGNFPSFPHDEVTDRNMLAFSFPAAGGVCEPLKSPQRAEADDPQDMA
CTPSGDSLETKEDQKMSPKATEETGQAQSGQANCQGLSPVSVASKNPQVPSDGGVRLNKSKTDLLVNDNP
DPAPLSPELQDFKCNICGYGYYGNDPTDLIKHFRKYHLGLHNRTRQDAELDSKILALHNMVQFSHSKDFQ
KVNRSVFSGVLQDINSSRPVLLNGTYDVQVTSGGTFIGIGRKTPDCQGNTKYFRCKFCNFTYMGNSSTEL
EQHFLQTHPNKIKASLPSSEVAKPSEKNSNKSIPALQSSDSGDLGKWQDKITVKAGDDTPVGYSVPIKPL
DSSRQNGTEATSYYWCKFCSFSCESSSSLKLLEHYGKQHGAVQSGGLNPELNDKLSRGSVINQNDLAKSS
EGETMTKTDKSSSGAKKKDFSSKGAEDNMVTSYNCQFCDFRYSKSHGPDVIVVGPLLRHYQQLHNIHKCT
IKHCPFCPRGLCSPEKHLGEITYPFACRKSNCSHCALLLLHLSPGAAGSSRVKHQCHQCSFTTPDVDVLL
FHYESVHESQASDVKQEANHLQGSDGQQSVKESKEHSCTKCDFITQVEEEISRHYRRAHSCYKCRQCSFT
AADTQSLLEHFNTVHCQEQDITTANGEEDGHAISTIKEEPKIDFRVYNLLTPDSKMGEPVSESVVKREKL
EEKDGLKEKVWTESSSDDLRNVTWRGADILRGSPSYTQASLGLLTPVSGTQEQTKTLRDSPNVEAAHLAR
PIYGLAVETKGFLQGAPAGGEKSGALPQQYPASGENKSKDESQSLLRRRRGSGVFCANCLTTKTSLWRKN
ANGGYVCNACGLYQKLHSTPRPLNIIKQNNGEQIIRRRTRKRLNPEALQAEQLNKQQRGSNEEQVNGSPL
ERRSEDHLTESHQREIPLPSLSKYEAQGSLTKSHSAQQPVLVSQTLDIHKRMQPLHIQIKSPQESTGDPG
NSSSVSEGKGSSERGSPIEKYMRPAKHPNYSPPGSPIEKYQYPLFGLPFVHNDFQSEADWLRFWSKYKLS
VPGNPHYLSHVPGLPNPCQNYVPYPTFNLPPHFSAVGSDNDIPLDLAIKHSRPGPTANGASKEKTKAPPN
VKNEGPLNVVKTEKVDRSTQDELSTKCVHCGIVFLDEVMYALHMSCHGDSGPFQCSICQHLCTDKYDFTT
HIQRGLHRNNAQVEKNGKPKE

FIGURE 9A

FIGURE 10

A. BCY1 cDNA Sequence

TGCAAGATTAAGGCCTTGAGGGCCAAGACCAACACCTACATCAAGACACCGGTGAGGGGCGAGGAACCAGTGTTCATG
GTGACAGGGCGACGGGAGGACGTGGCCACAGCCCGGCGGGAAATCATCTCAGCAGCGGAGCACTTCTCCATGATCCGT
GCCTCCCGCAACAAGTCAGGCGCCGCCTTTGGTGTGGCTCCTGCTCTGCCCGGCCAGGTGACCATCCGTGTGCGGGTG
CCCTACCGCGTGGTGGGGCTGGTGGTGGGCCCCAAAGGGGCAACCATCAAGCGCATCCAGCAGCAAACCAACACATAC
ATTATCACACCAAGCCGTGACCGCGACCCCGTGTTCGAGATCACGGGTGCCCCAGGCAACGTGGAGCGTGCGCGCGAG
GAGATCGAGACGCACATCGCGGTGCGCACTGGCAAGATCCTCGAGTACAACAATGAAAACGACTTCCTGGCGGGGAGC
CCCGACGCAGCAATCGATAGCCGCTACTCCGACGCCTGGCGGGTGCACCAGCCCGGCTGCAAGCCCCTCTCCACCTTC
CGGCAGAACAGCCTGGGCTGCATCGGCGAGTGCGGAGTGGACTCTGGCTTTGAGGCCCCACGCCTGGGTGAGCAGGGC
GGGGACTTTGGCTACGGCGGGTACCTCTTTCCGGGCTATGGCGTGGGCAAGCAGGATGTGTACTACGGCGTGGCCGAG
ACTAGCCCCCCGCTGTGGGCGGGCCAGGAGAACGCCACGCCCACCTCCGTGCTCTTCTCCTCYKCCTCCTCCTCCTCC
TCCTCTTCCGCCAAGGCCCGCGCTGGGCCCCCGGGCGCACACCGCTCCCCTGCCACTTCCGCGGGACCCGAGCTGGCC
GGACTCCCGAGGCGCCCCCCGGGAGAGCCGCTCCRGGGCTTCTCTAAACTTGGTGGGGGCGGCCTGCGGAGCCCCGCA
GCCGGCGGGCGGGATTGCATGGTCTGCTTTGAGAGCGAAGTGACTGCCGCCCTTGTGCCCTGCGGACACAACCTGTTC
TGCATGGAGTGTGCAGTACGCATCTGCGAGAGGACGGACCCAGAGTGTCCCGTCTGCCACATCACAGCCACGCAAGCC
ATCCGAATATTCTCCTAAGCCCCGTGCCCCATGCCTCCGGGGCCCACTCCACTGGGCCCACCCTGGACCTGTTTTCCA
CTAAAGCCTTTTGGAAAGCGGTGATTTGAGGGGCAAGGTGCTTAGAGATACTCGCTCGCTGGGGAAGGGGGGAGGGAG
GCAGTGGTGGCTGGAGGGTGCGCCACTTTCAGAGCCTCTGGTCACCCTGTCCTGGAAAGATTGGGAGGGGGCCAGACT
GAAAATTTTACTAGAGTTACAACTCTGATACCTCAACACACCCTTAAATCTGGAAGCAGCTAAGAGAAACTTTTGTTT
TGCCAGAGGTGGCCACTAAGGCATTCTGACGCCCTCTGCCCACCTCCCCCGCTGTGTGTCACTCCACCCCTTCTTCCG
AGGAGGGGGTGGGTAAAAGGGAGAGGGAGAATTACCACCTGTATCTAGAGGTGCTCTTTGCAATCCCTAAGCCCTCTG
GTCCTGACCTCCGACCTCCCAGCTCTGTCTTGTTCCTTGTCTTTGTCTTTCTTCCCTTCCCCCTGCCCCTGCCCCTAC
CAGCCCAGCTTTGGGGACACCATCCTTCTGGGGAGAAGTAGGGGGAGGAATATTTGGATGGTCCCTCCATTCCTCTTC
AGGCATCTGGAGGCCCTCTCCCCCACTCCTCCAAAGAAACATCTCAAATTATTGATGGAATGTATCCCCATTCTCAGT
GAAAATGTGAGGAGGGGACTAATACTGGGGTAAAGGGTCAAACCCCCACCTTCATCACTATGGGCATTATATTTAGGG
AGTAGTTCTTGGGCTGGATTTTCTGGTTGTGGAAGTGGGGGCGCCAGAGTAGTGTGTCTGCTATTTAAAGGAGCAGGA
AAGGGCGTGAGGCAGGAGGAGAGACTGGTGGAGGGAAGAGCTGCTCCTCCCATGCAGTGCCCGACTCCCTGCACCCCT
CTCAACCTGACCTGAACCTTTATTGAATCCTTATTAGCTTGAATCCTTATTAGCTTGAATCCTCCATGCAAATCATGG
AGTCTGTGTCCCACCTGATGTGGTTGAGGAGAAGCCAGGTCTTCAAAGAGGGGTCAGCCTGGGGCAAAGCAGGACTGG
GGGGAGGTGGGCAGCAGGGCCTATTCTGAGAATCACATATTGTTACAGGCCTTGCACCCCCTTTGCTGCTTCCCTCCT
GCTCATTTGGGGCTGCCACCAGCTCTCCACCCTCCTGGTTCCGCTGGCCGGGCCAAGAGAGGATGGAGGGATGGGAGT
CCCAGGAGATCCTTGTAAATAGTGGGGTGGGACTGTTCTGAGTGATCACCCGAGCACTTAAAGCTCCAGAGTCCCATT
CTTCCTGGATGGAGCAGGTGGAGGTGCAGAGGGGATTTCCTCCTCTCCTTCCTCCTGTCGAGAATTAACACCTCTCCA
CAGCCTTCCCCTCCAGAACACCAGCCAGGGAGGGGTGGGGAAGGAGGTCACAGCCAAGAAAACTGCCCTGTGACGACT
TCCCTCCTTCCCGCCTATGTGAGCCATCCTGAGATGTCTGTACAATAGAAACCAAACCAAATGGGCACCCTCGGTTGC
CGGGGGGCAGGTGGGGAGGGGGGTGGGAAGAAGGGATGTCTGTCTGTCGTCCCCCTCCCCCCTCTCCACTCTTTACCCA
CAAAGGCAGAAGACTGTTACACTAGGGGGCTCAGCAAATTCAATCCCACCCTTACCAATTGAGCCAAACCTAGAAACA
AACACAAAACACGAATAGTGAGAGACAAAATAGAGGAGAGAAAGAGAGCATGAGAGGGAGCGAGACAGGCGACCAACA
CAGAGGAGAGAAAACAAAAATAGCAAAAAAAAAAAAAAAAAA

B. BCY1 Amino Acid Sequence

MAELRLKGSS NTTECVPVPT SEHVAEIVGR QGCKIKALRA KTNTYIKTPV RGEEPVFMVT
GRREDVATAR REIISAAEHF SMIRASRNKS GAAFGVAPAL PGQVTIRVRV PYRVVGLVVG
PKGATIKRIQ QQTNTYIITP SRDRDPVFEI TGAPGNVERA REEIETHIAV RTGKILEYNN
ENDFLAGSPD AAIDSRYSDA WRVHQPGCKP LSTFRQNSLG CIGECGVDSG FEAPRLGEQG
GDFGYGGYLF PGYGVGKQDV YYGVAETSPP LWAGQENATP TSVLFSSASS SSSSSAKARA
GPPGAHRSPA TSAGPELAGL PRRPPGEPLQ GFSKLGGGGL RSPGGGRDCM
VCFESEVTAA LVPCGHNLFC MECAVRICER TDPECPVCHI TAAQAIRIFS

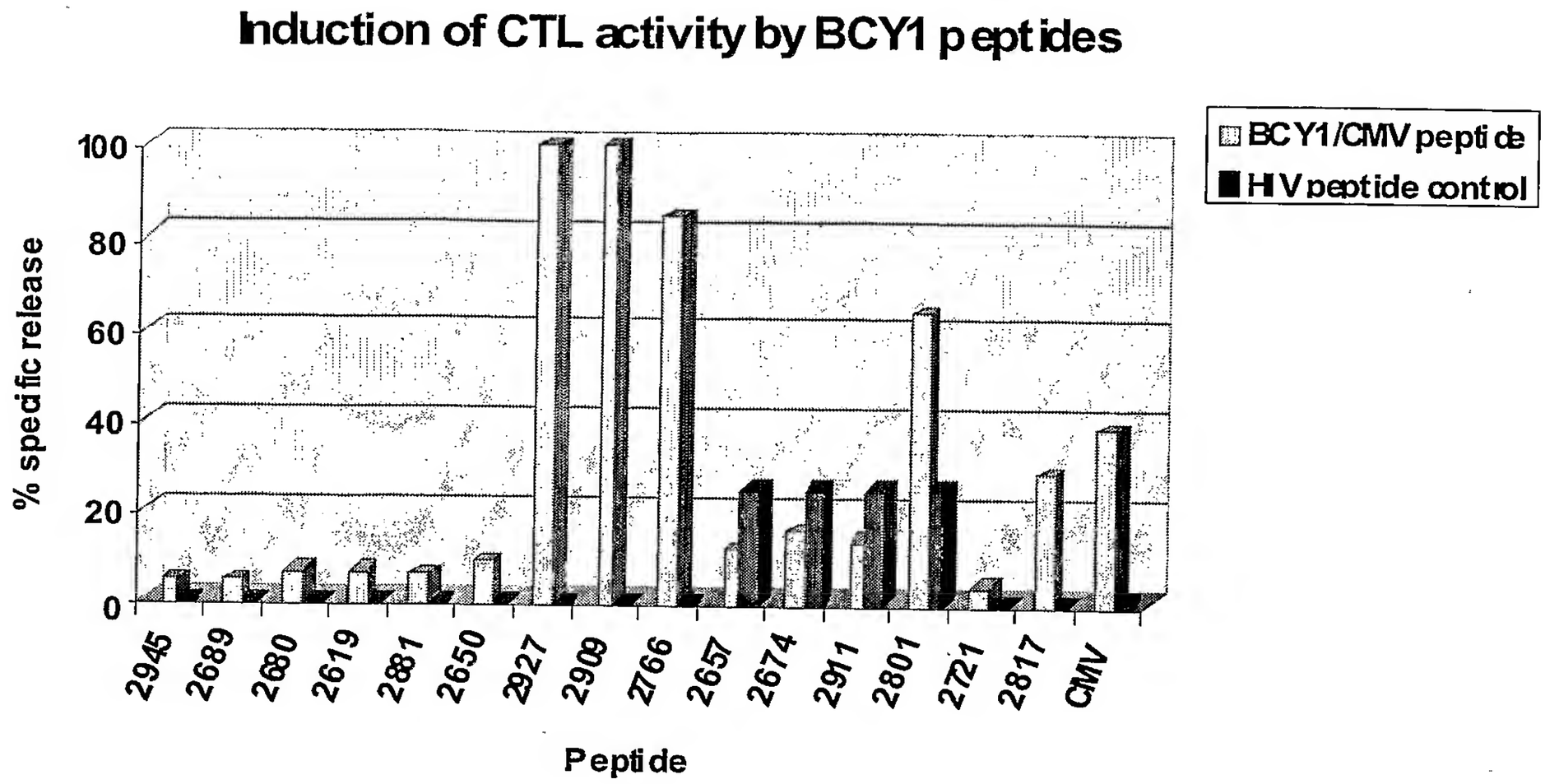
Induction of CTL activity by BCY1 peptides
BCY1/CMV peptide
HIV peptide control
% specific release
100
80
60
40
20
0
2945
2689
2680
2619
2881
2650
2927
2909
2766
2657
2674
2911
2801
2721
2817
CMV
Peptide

FIGURE 11

FIGURE 12

ATGACAAAGAGGAAGAAGACCATCAACCTTAATATACAAGACGCCCAGAAGAGGACTGCTCTACACTGGGCCTGTGTC
AATGGCCATGAGGAAGTAGTAACATTTCTGGTAGACAGAAAGTGCCAGCTTGACGTCCTTGATGGCGAACACAGGACA
CCTCTGATGAAGGCTCTACAATGCCATCAGGAGGCTTGTGCAAATATTCTGATAGATTCTGGTGCCGATATAAATCTC
GTAGATGTGTATGGCAACATGGCTCTCCATTATGCTGTTTATAGTGAGATTTTGTCAGTGGTGGCAAAACTGCTGTCC
CATGGTGCAGTCATCGAAGTGCACAACAAGGCTAGCCTCACACCACTTTTACTATCCATAACGAAAAGAAGTGAGCAA
ATTGTGGAATTTTTGCTGATAAAAAATGCAAATGCGAATGCAGTTAATAAGTATAAATGCACAGCCCTCATGCTTGCT
GTATGTCATGGATCATCAGAGATAGTTGGCATGCTTCTTCAGCAAAATGTTGACGTCTTTGCTGCAGATATATGTGGA
GTAACTGCAGAACATTATGCTGTTACTTGTGGATTTCATCACATTCATGAACAAATTATGGAATATATACGAAAATTA
TCTAAAAATCATCAAAATACCAATCCAGAAGGAACATCTGCAGGAACACCTGATGAGGCTGCACCCTTGGCGGAAAGA
ACACCTGACACAGCTGAAAGCTTGGTGGAAAAAACACCTGATGAGGCTGCACCCTTGGTGGAAAGAACACCTGACACG
GCTGAAAGCTTGGTGGAAAAAACACCTGATGAGGCTGCATCCTTGGTGGAGGGAACATCTGACAAAATTCAATGTTTG
GAGAAAGCGACATCTGGAAAGTTCGAACAGTCAGCAGAAGAAACACCTAGGGAAATTACGAGTCCTGCAAAAGAAACA
TCTGAGAAATTTACGTGGCCAGCAAAAGGAAGACCTAGGAAGATCGCATGGGAGAAAAAAGAAGACACACCTAGGGAA
ATTATGAGTCCCGCAAAAGAAACATCTGAGAAATTTACGTGGGCAGCAAAAGGAAGACCTAGGAAGATCGCATGGGAG
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AAACAAGAGGAAGATGAAGAATATTCTTGTGATTCTCGGAGTCTCTTTGAGAGTTCTGCAAAGATTCAAGTGTGTATA
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CCTGCCATTGAAATGCAAAACTCTGTTCCAAATAAAGCCTTTGAATTGAAGAATGAACAAACATTGAGAGCAGATCCG
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CAGAAGGATGTGTGTTTACCCAAGGCTACACATCAAAAAGAAATAGATAAAATAAATGGAAAATTAGAAGAGTCTCCT
AATAAAGATGGTCTTCTGAAGGCTACCTGCGGAATGAAAGTTTCTATTCCAACTAAAGCCTTAGAATTGAAGGACATG
CAAACTTTCAAAGCGGAGCCTCCGGGGAAGCCATCTGCCTTCGAGCCTGCCACTGAAATGCAAAAGTCTGTCCCAAAT
AAAGCCTTGGAATTGAAAAATGAACAAACATGGAGAGCAGATGAGATACTCCCATCAGAATCCAAACAAAAGGACTAT
GAAGAAAATTCTTGGGATACTGAGAGTCTCTGTGAGACTGTTTCACAGAAGGATGTGTGTTTACCCAAGGCTGCGCAT
CAAAAAGAAATAGATAAAATAAATGGAAAATTAGAAGGGTCTCCTGTTAAAGATGGTCTTCTGAAGGCTAACTGCGGA
ATGAAAGTTTCTATTCCAACTAAAGCCTTAGAATTGATGGACATGCAAACTTTCAAAGCAGAGCCTCCCGAGAAGCCA
TCTGCCTTCGAGCCTGCCATTGAAATGCAAAAGTCTGTTCCAAATAAAGCCTTGGAATTGAAGAATGAACAAACATTG
AGAGCAGATGAGATACTCCCATCAGAATCCAAACAAAAGGACTATGAAGAAAGTTCTTGGGATTCTGAGAGTCTCTGT
GAGACTGTTTCACAGAAGGATGTGTGTTTACCCAAGGCTACACATCAAAAAGAAATAGATAAAATAAATGGAAAATTA
GAAGAGTCTCCTGATAATGATGGTTTTCTGAAGGCTCCCTGCAGAATGAAAGTTTCTATTCCAACTAAAGCCTTAGAA
TTGATGGACATGCAAACTTTCAAAGCAGAGCCTCCCGAGAAGCCATCTGCCTTCGAGCCTGCCATTGAAATGCAAAAG
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CAAAAGAAGGTTGAAGAAAATTCTTGGGATTCTGAGAGTCTCCGTGAGACTGTTTCACAGAAGGATGTGTGTGTACCC
AAGGCTACACATCAAAAAGAAATGGATAAAATAAGTGGAAAATTAGAAGATTCAACTAGCCTATCAAAAATCTTGGAT
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AAAAAGAAGTTTTGTGTACTGAAAAAGAAACTGTCAGAAGCAAAAGAAATAAAATCACAGTTAGAGAACCAAAAAGTT
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AATGAAAAAATTAGGGAAGAATTAGGAAGAATCGAAGAGCAGCATAGGAAAGAGTTAGAAGTGAAACAACAACTTGAA
CAGGCTCTCAGAATACAAGATATAGAATTGAAGAGTGTAGAAAGTAATTTGAATCAGGTTTCTCACACTCATGAAAAT
GAAAATTATCTCTTACATGAAAATTGCATGTTGAAAAAGGAAATTGCCATGCTAAAACTGGAAATAGCCACACTGAAA
CACCAATACCAGGAAAAGGAAAATAAATACTTTGAGGACATTAAGATTTTAAAAGAAAAGAATGCTGAACTTCAGATG
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AACACAATGCTCACTTCTAAATTGAAGGAAAAACAAGACAAAGAAATACTAGAGGCAGAAATTGAATCACACCATCCT
AGACTGGCTTCTGCTGTACAAGACCATGATCAAATTGTGACATCAAGAAAAAGTCAAGAACCTGCTTTCCACATTGCA
GGAGATGCTTGTTTGCAAAGAAAAATGAATGTTGATGTGAGTAGTACGATATATAACAATGAGGTGCTCCATCAACCA
CTTTCTGAAGCTCAAAGGAAATCCAAAAGCCTAAAAATTAATCTCAATTATGCAGGAGATGCTCTAAGAGAAAATACA
TTGGTTTCAGAACATGCACAAAGAGACCAACGTGAAACACAGTGTCAAATGAAGGAAGCTGAACACATGTATCAAAAC
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ATGTGGCTTCAACAGCAATTAGTTCATGCACATAAGAAAGCTGACAACAAAAGCAAGATAACAATTGATATTCATTTT
CTTGAGAGGAAAATGCAACATCATCTCCTAAAAGAGAAAAATGAGGAGATATTTAATTACAATAACCATTTAAAAAAC
CGTATATATCAATATGAAAAAGAGAAAGCAGAAACAGAAAACTCATGA

FIGURE 13

MTKRKKTINLNIQDAQKRTALHWACVNGHEEVVTFLVDRKCQLDVLDGEHRTPLMKALQCHQEACANILIDSGADINL
VDVYGNMALHYAVYSEILSVVAKLLSHGAVIEVHNKASLTPLLLSITKRSEQIVEFLLIKNANANAVNKYKCTALMLA
VCHGSSEIVGMLLQQNVDVFAADICGVTAEHYAVTCGFHHIHEQIMEYIRKLSKNHQNTNPEGTSAGTPDEAAPLAER
TPDTAESLVEKTPDEAAPLVERTPDTAESLVEKTPDEAASLVEGTSDKIQCLEKATSGKFEQSAEETPREITSPAKET
SEKFTWPAKGRPRKIAWEKKEDTPREIMSPAKETSEKFTWAAKGRPRKIAWEKKETPVKTGCVARVTSNKTKVLEKGR
SKMIACPTKESSTKASANDQRFPSESKQEEDEEYSCDSRSLFESSAKIQVCIPESIYQKVMEINREVEEPPKKPSAFK
PAIEMQNSVPNKAFELKNEQTLRADPMFPPESKQKDYEENSWDSESLCETVSQKDVCLPKATHQKEIDKINGKLEESP
NKDGLLKATCGMKVSIPTKALELKDMQTFKAEPPGKPSAFEPATEMQKSVPNKALELKNEQTWRADEILPSESKQKDY
EENSWDTESLCETVSQKDVCLPKAAHQKEIDKINGKLEGSPVKDGLLKANCGMKVSIPTKALELMDMQTFKAEPPEKP
SAFEPAIEMQKSVPNKALELKNEQTLRADEILPSESKQKDYEESSWDSESLCETVSQKDVCLPKATHQKEIDKINGKL
EESPDNDGFLKAPCRMKVSIPTKALELMDMQTFKAEPPEKPSAFEPAIEMQKSVPNKALELKNEQTLRADQMFPSESK
QKKVEENSWDSESLRETVSQKDVCVPKATHQKEMDKISGKLEDSTSLSKILDTVHSCERARELQKDHCEQRTGKMEQM
KKKFCVLKKKLSEAKEIKSQLENQKVKWEQELCSVRLTLNQEEEKRRNADILNEKIREELGRIEEQHRKELEVKQQLE
QALRIQDIELKSVESNLNQVSHTHENENYLLHENCMLKKEIAMLKLEIATLKHQYQEKENKYFEDIKILKEKNAELQM
TLKLKEESLTKRASQYSGQLKVLIAENTMLTSKLKEKQDKEILEAEIESHHPRLASAVQDHDQIVTSRKSQEPAFHIA
GDACLQRKMNVDVSSTIYNNEVLHQPLSEAQRKSKSLKINLNYAGDALRENTLVSEHAQRDQRETQCQMKEAEHMYQN
EQDNVNKHTEQQESLDQKLFQLQSKNMWLQQQLVHAHKKADNKSKITIDIHFLERKMQHHLLKEKNEEIFNYNNHLKN
RIYQYEKEKAETENS

FIGURE 14A

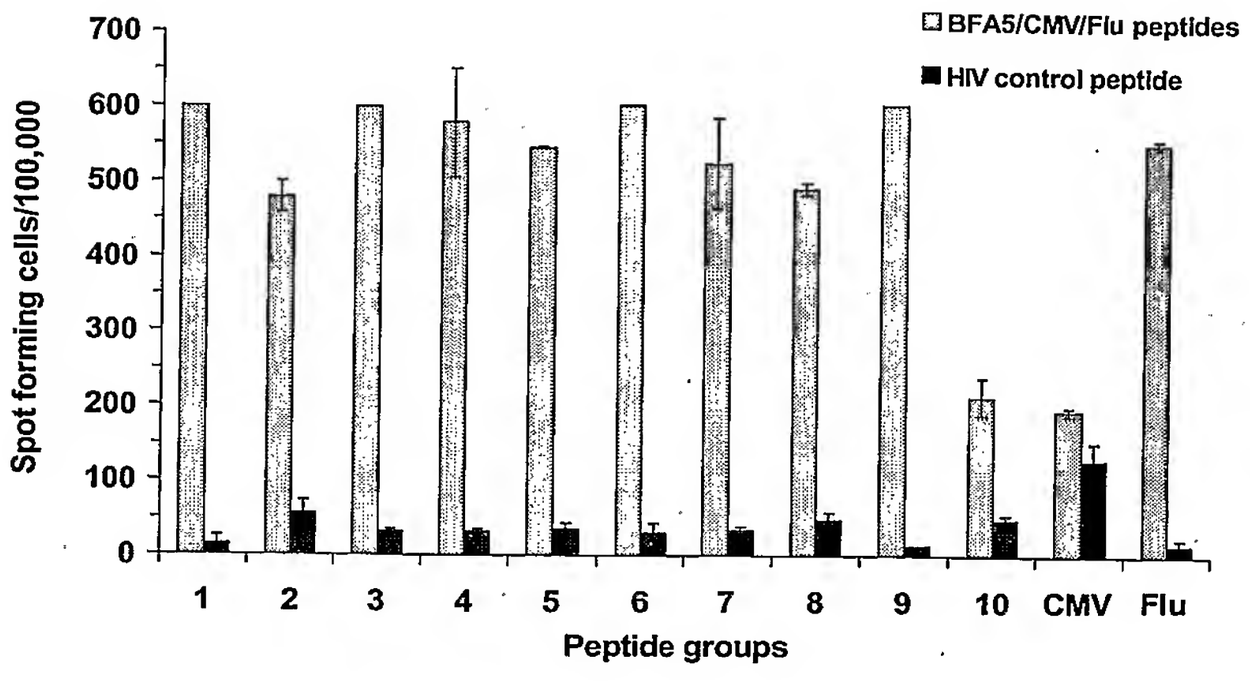
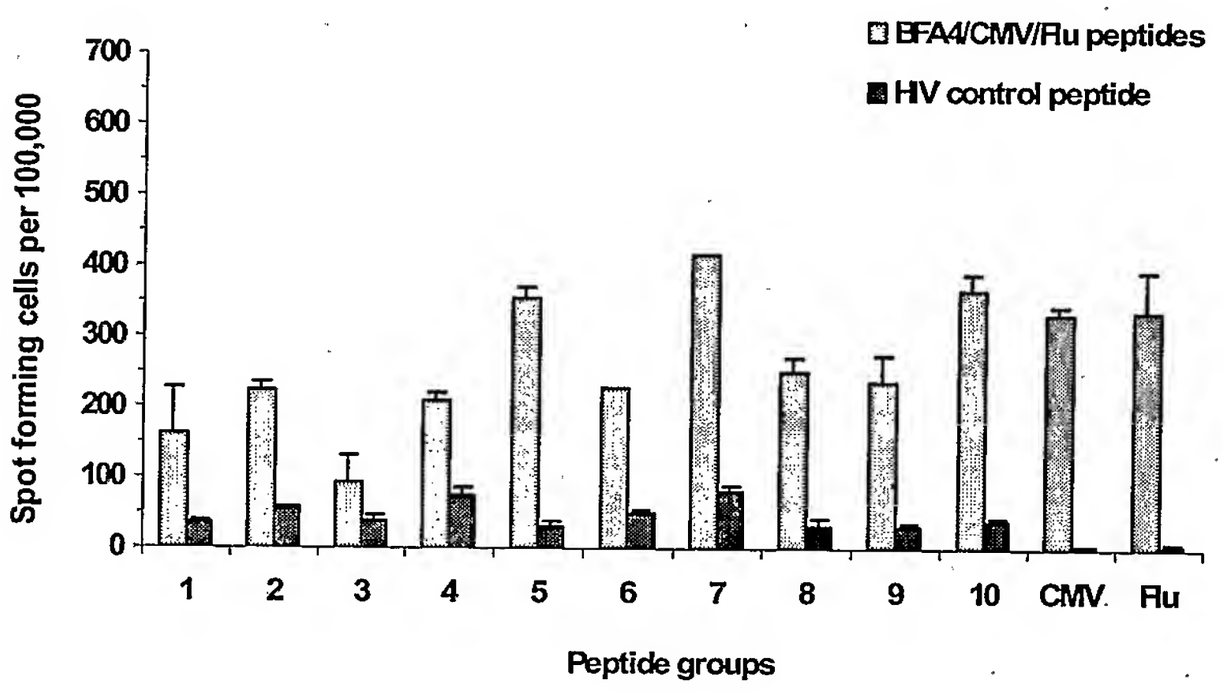


FIGURE 14B



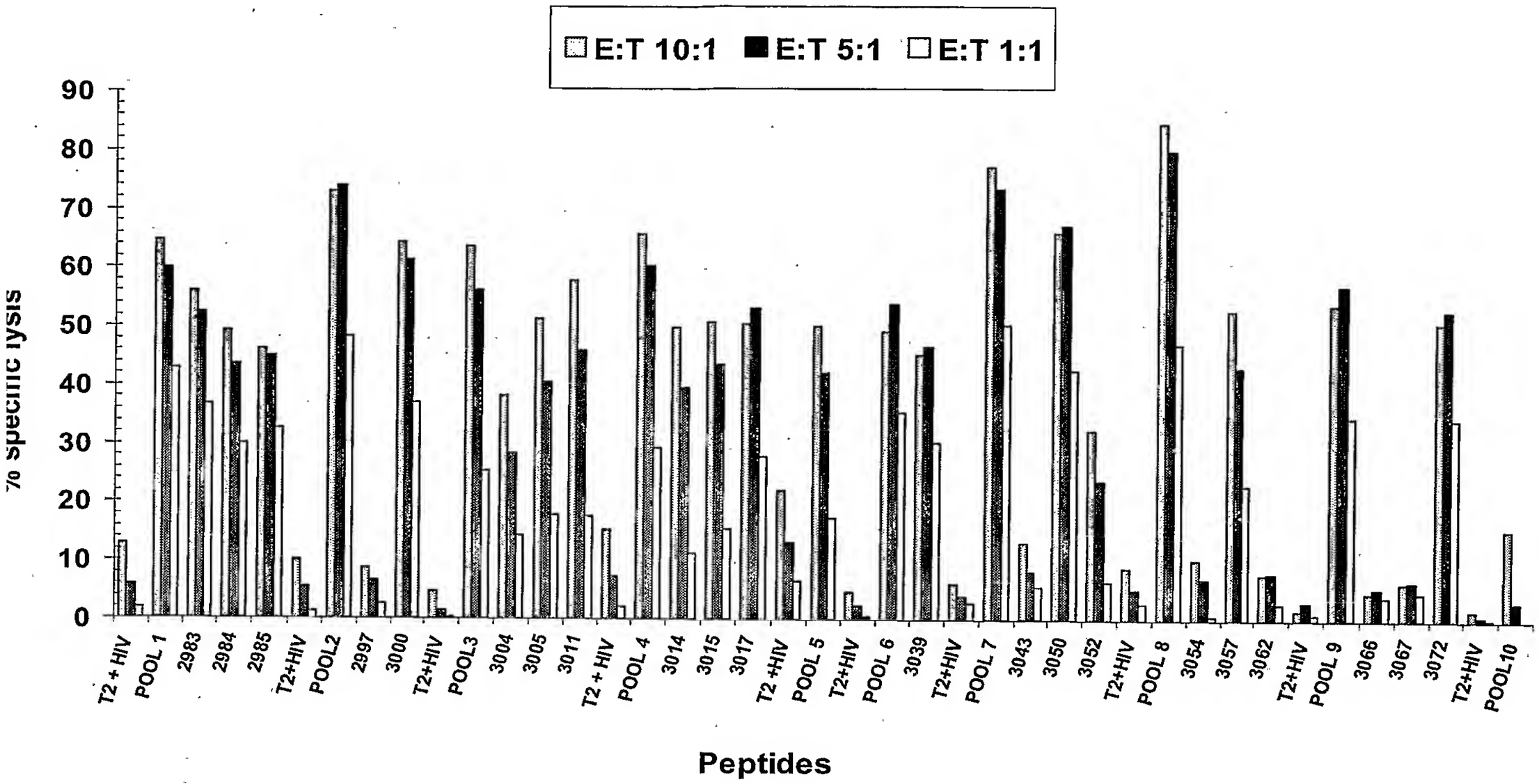

E:T 10:1
E:T 5:1
E:T 1:1
% specific lysis
90
80
70
60
50
40
30
20
10
0
T2 + HIV
POOL 1
2983
2984
2985
T2+HIV
POOL2
2997
3000
T2+HIV
POOL3
3004
3005
3011
T2 + HIV
POOL 4
3014
3015
3017
T2 +HIV
POOL 5
T2+HIV
POOL 6
3039
T2+HIV
POOL 7
3043
3050
3052
T2+HIV
POOL 8
3054
3057
3062
T2+HIV
POOL 9
3066
3067
3072
T2+HIV
POOL10
Peptides

FIGURE 14C

FIGURE 15

A. BCZ4 cDNA

ATGGACATTGAAGCATATCTTGAAAGAATTGGCTATAAGAAGTCTAGGAACAAATTGGACTTGGAAACATTAACTGAC
ATTCTTCAACACCAGATCCGAGCTGTTCCCTTTGAGAACCTTAACATCCATTGTGGGGATGCCATGGACTTAGGCTTA
GAGGCCATTTTTGATCAAGTTGTGAGAAGAAATCGGGGTGGATGGTGTCTCCAGGTCAATCATCTTCTGTACTGGGCT
CTGACCACTATTGGTTTTGAGACCACGATGTTGGGAGGGTATGTTTACAGCACTCCAGCCAAAAAATACAGCACTGGC
ATGATTCACCTTCTCCTGCAGGTGACCATTGATGGCAGGAACTACATTGTCGATGCTGGGTTTGGACGCTCATACCAG
ATGTGGCAGCCTCTGGAGTTAATTTCTGGGAAGGATCAGCCTCAGGTGCCTTGTGTCTTCCGTTTGACGGAAGAGAAT
GGATTCTGGTATCTAGACCAAATCAGAAGGGAACAGTACATTCCAAATGAAGAATTTCTTCATTCTGATCTCCTAGAA
GACAGCAAATACCGAAAAATCTACTCCTTTACTCTTAAGCCTCGAACAATTGAAGATTTTGAGTCTATGAATACATAC
CTGCAGACATCTCCATCATCTGTGTTTACTAGTAAATCATTTTGTTCCTTGCAGACCCCAGATGGGGTTCACTGTTTG
GTGGGCTTCACCCTCACCCATAGGAGATTCAATTATAAGGACAATACAGATCTAATAGAGTTCAAGACTCTGAGTGAG
GAAGAAATAGAAAAAGTGCTGAAAAATATATTTAATATTTCCTTGCAGAGAAAGCTTGTGCCCAAACATGGTGATAGA
TTTTTTACTATTTAG

B. BCZ4 Amino Acid Sequence

MDIEAYLERIGYKKSRNKLDLETLTDILQHQIRAVPFENLNIHCGDAMDLGLEAIFDQVVRRNRGGWCLQVNHLLYWA
LTTIGFETTMLGGYVYSTPAKKYSTGMIHLLLQVTIDGRNYIVDAGFGRSYQMWQPLELISGKDQPQVPCVFRLTEEN
GFWYLDQIRREQYIPNEEFLHSDLLEDSKYRKIYSFTLKPRTIEDFESMNTYLQTSPSSVFTSKSFCSLQTPDGVHCL
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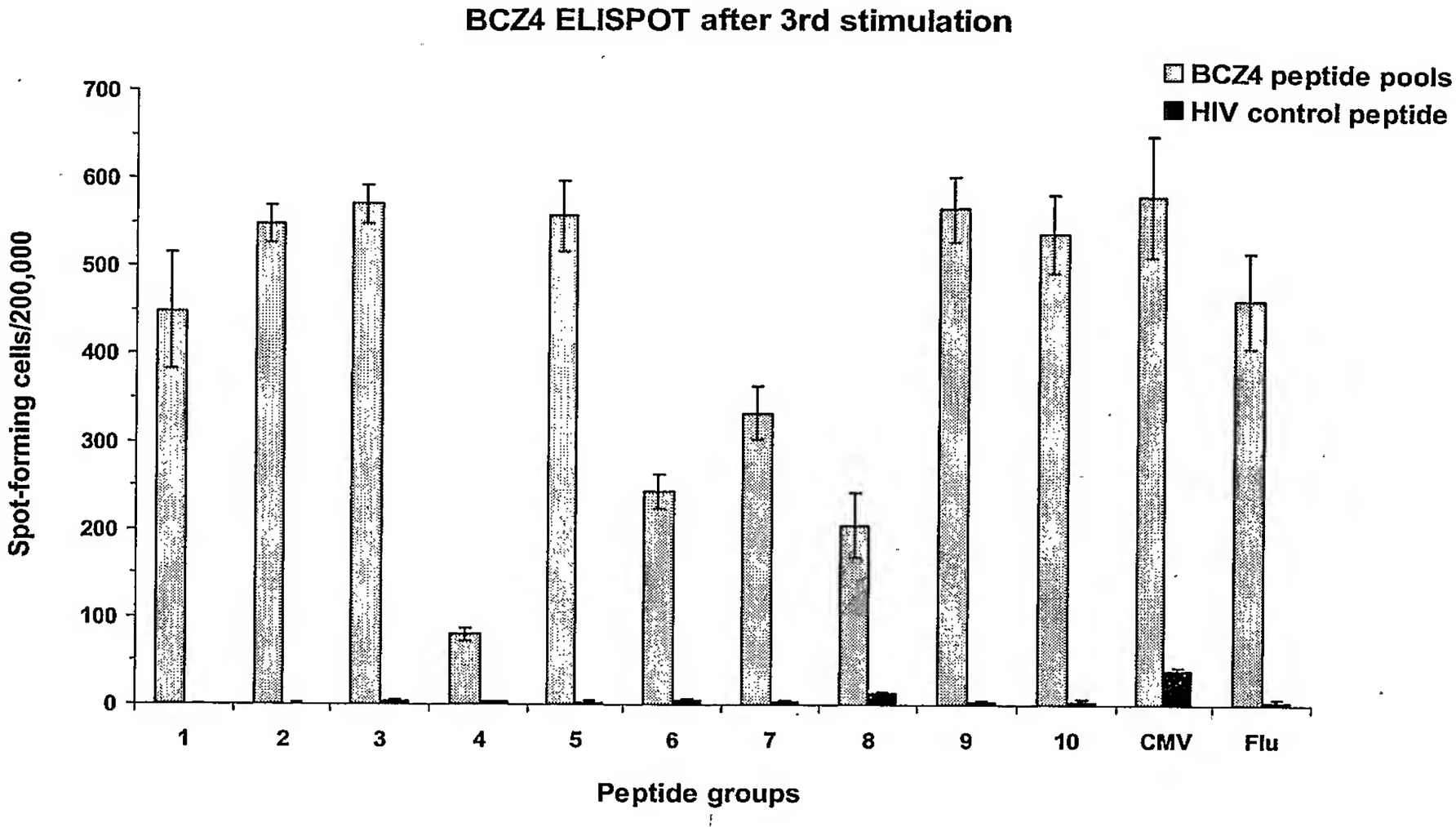
BCZ4 ELISPOT after 3rd stimulation
BCZ4 peptide pools
HIV control peptide
Spot-forming cells/200,000
700
600
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
10
CMV
Flu
Peptide groups

FIGURE 16A

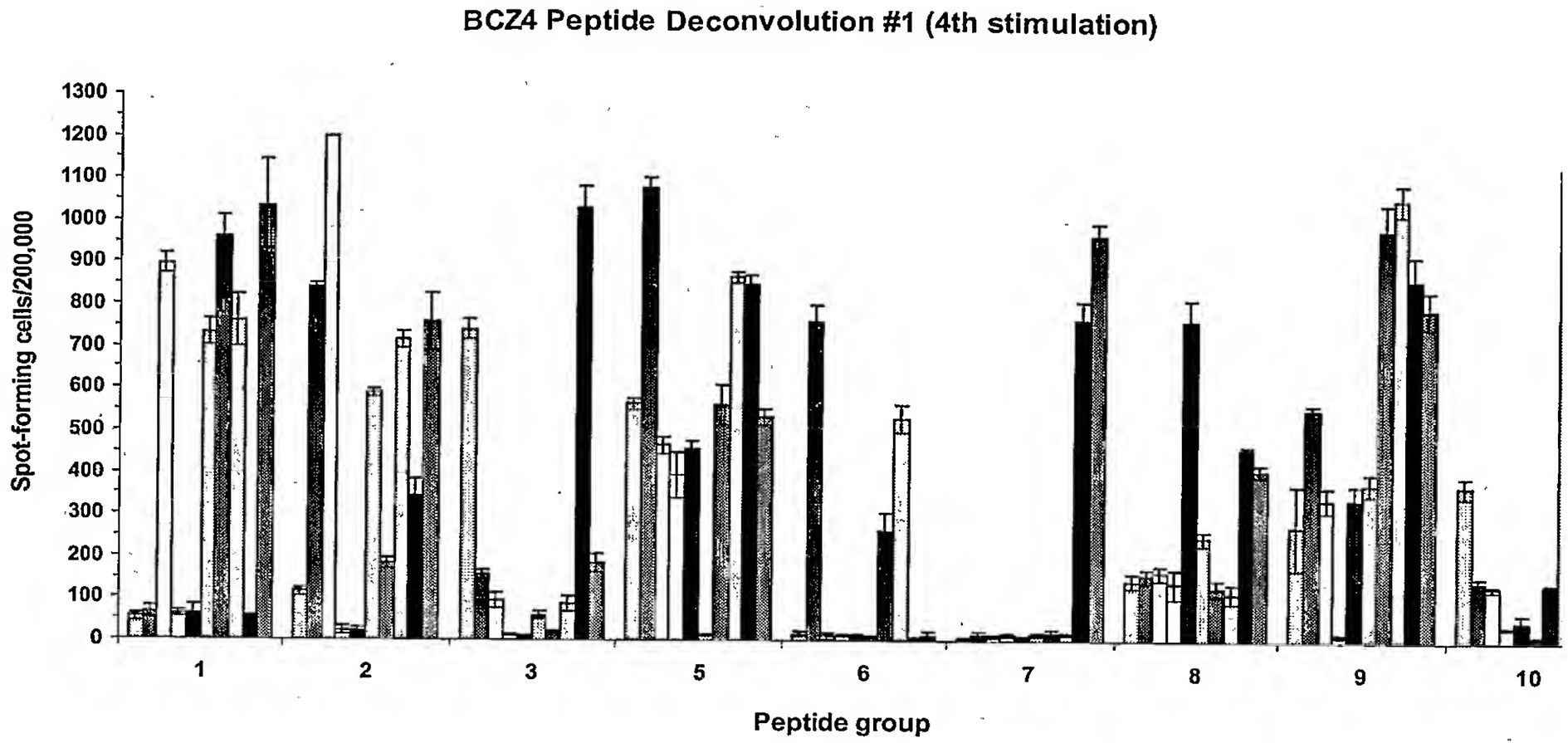
BCZ4 Peptide Deconvolution #1 (4th stimulation)
Spot-forming cells/200,000
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
1
2
3
5
6
7
8
9
10
Peptide group

FIGURE 16B

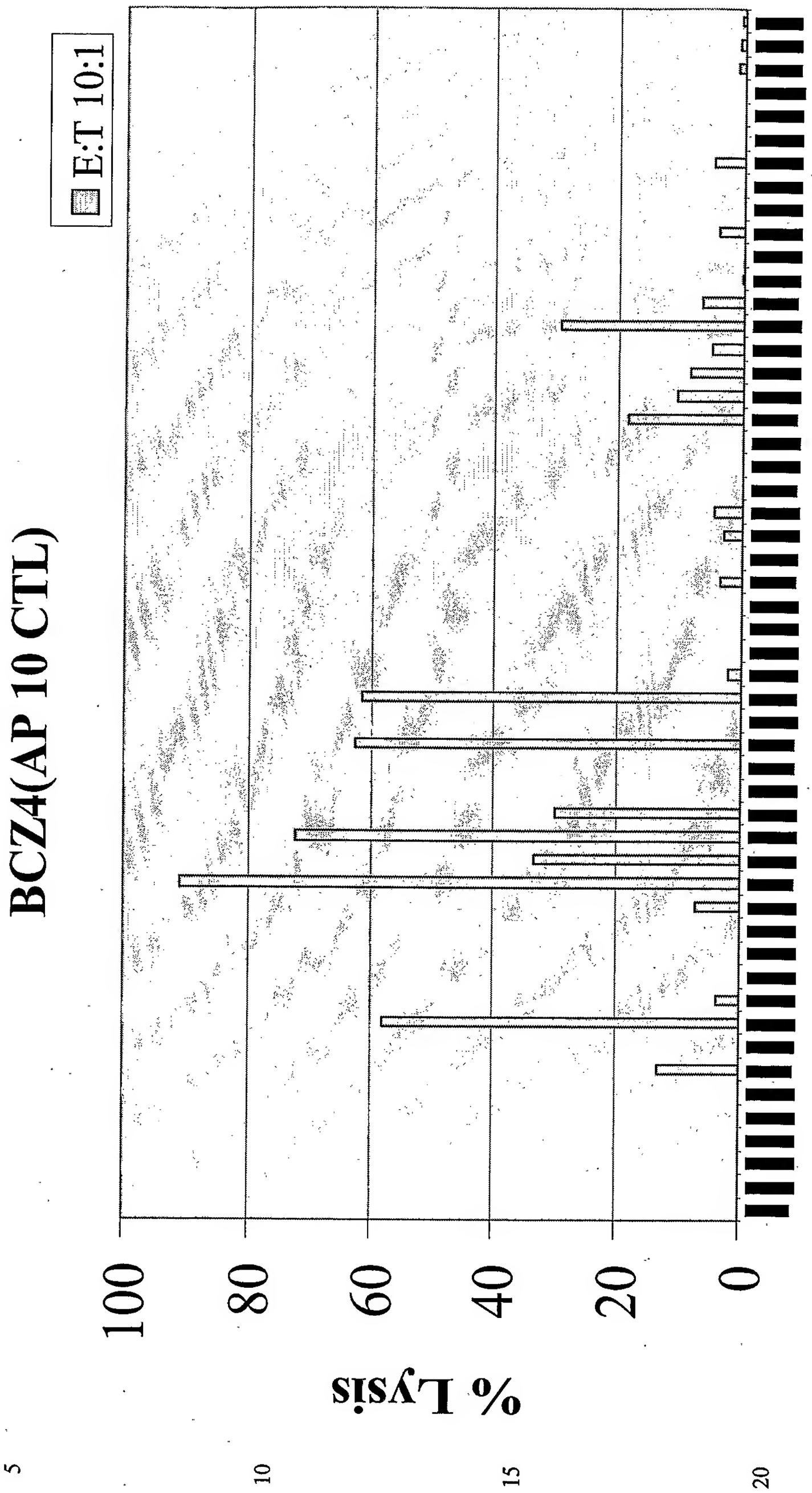

FIGURE 16C

FIGURE 17

A. BFY3 cDNA

```
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GCTGGGCACTGTAGGTCAATCTCCCTACACGAGCGCCCCGCCGCTGTCCCACACCCCCAATGCCGACTTCCAGCCCCCATACTTCCCCC
CACCCTACCAGCCTATCTACCCCCAGTCGCAAGATCCTTACTCCCACGTCAACGACCCCTACAGCCTGAACCCCCTGCACGCCCAGCCG
CAGCCGCAGCACCCAGGCTGGCCCGGCCAGAGGCAGAGCCAGGAGTCTGGGCTCCTGCACACGCACCGGGGGCTGCCTCACCAGCTGTC
GGGCCTGGATCCTCGCAGGGACTACAGGCGGCACGAGGACCTCCTGCACGGCCCACACGCGCTCAGCTCAGGACTCGGAGACCTCTCGA
TCCACTCCTTACCTCACGCCATCGAGGAGGTCCCGCATGTAGAAGACCCGGGTATTAACATCCCAGATCAAACTGTAATTAAGAAAGGC
CCCGTGTCCCTGTCCAAGTCCAACAGCAATGCCGTCTCCGCCATCCCTATTAACAAGGACAACCTCTTCGGCGGCGTGGTGAACCCCAA
CGAAGTCTTCTGTTCAGTTCCGGGTCGCCTCTCGCTCCTCAGCTCCACCTCGAAGTACAAGGTCACGGTGGCGGAAGTGCAGCGGCGGC
TCTCACCACCCGAGTGTCTCAACGCGTCGCTGCTGGGCGGAGTGCTCCGGAGGGCGAAGTCTAAAAATGGAGGAAGATCTTTAAGAGAA
AAACTGGACAAAATAGGATTAAATCTGCCTGCAGGGAGACGTAAAGCTGCCAACGTTACCCTGCTCACATCACTAGTAGAGGGAGAAGC
TGTCCACCTAGCCAGGGACTTTGGGTACGTGTGCGAAACCGAATTTCCTGCCAAAGCAGTAGCTGAATTTCTCAACCGACAACATTCCG
ATCCCAATGAGCAAGTGACAAGAAAAAACATGCTCCTGGCTACAAAACAGATATGCAAAGAGTTCACCGACCTGCTGGCTCAGGACCGA
TCTCCCCTGGGGAACTCACGGCCCAACCCCATCCTGGAGCCCGGCATCCAGAGCTGCTTGACCCACTTCAACCTCATCTCCCACGGCTT
CGGCAGCCCCGCGGTGTGTGCCGCGGTCACGGCCCTGCAGAACTATCTCACCGAGGCCCTCAAGGCCATGGACAAAATGTACCTCAGCA
ACAACCCCAACAGCCACACGGACAACAACGCCAAAAGCAGTGACAAAGAGGAGAAGCACAGAAAGTGA
```

B. BFY3 Amino Acid

```
MLWKLTDNIK YEDCEDRHDG TSNGTARLPQ LGTVGQSPYT SAPPLSHTPN
ADFQPPYFPP PYQPIYPQSQ DPYSHVNDPY SLNPLHAQPQ PQHPGWPGQR
QSQESGLLHT HRGLPHQLSG LDPRRDYRRH EDLLHGPHAL SSGLGDLSIH
SLPHAIEEVP HVEDPGINIP DQTVIKKGPV SLSKSNSNAV SAIPINKDNL
FGGVVNPNEV FCSVPGRLSL LSSTSKYKVT VAEVQRRLSP PECLNASLLG
GVLRRAKSKN GGRSLREKLD KIGLNLPAGR RKAANVTLLT SLVEGEAVHL
ARDFGYVCET EFPAKAVAEF LNRQHSDPNE QVTRKNMLLA TKQICKEFTD
LLAQDRSPLG NSRPNPILEP GIQSCLTHFN LISHGFGSPA VCAAVTALQN
YLTEALKAMD KMYLSNNPNS HTDNNAKSSD KEEKHRK
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FIGURE 18A

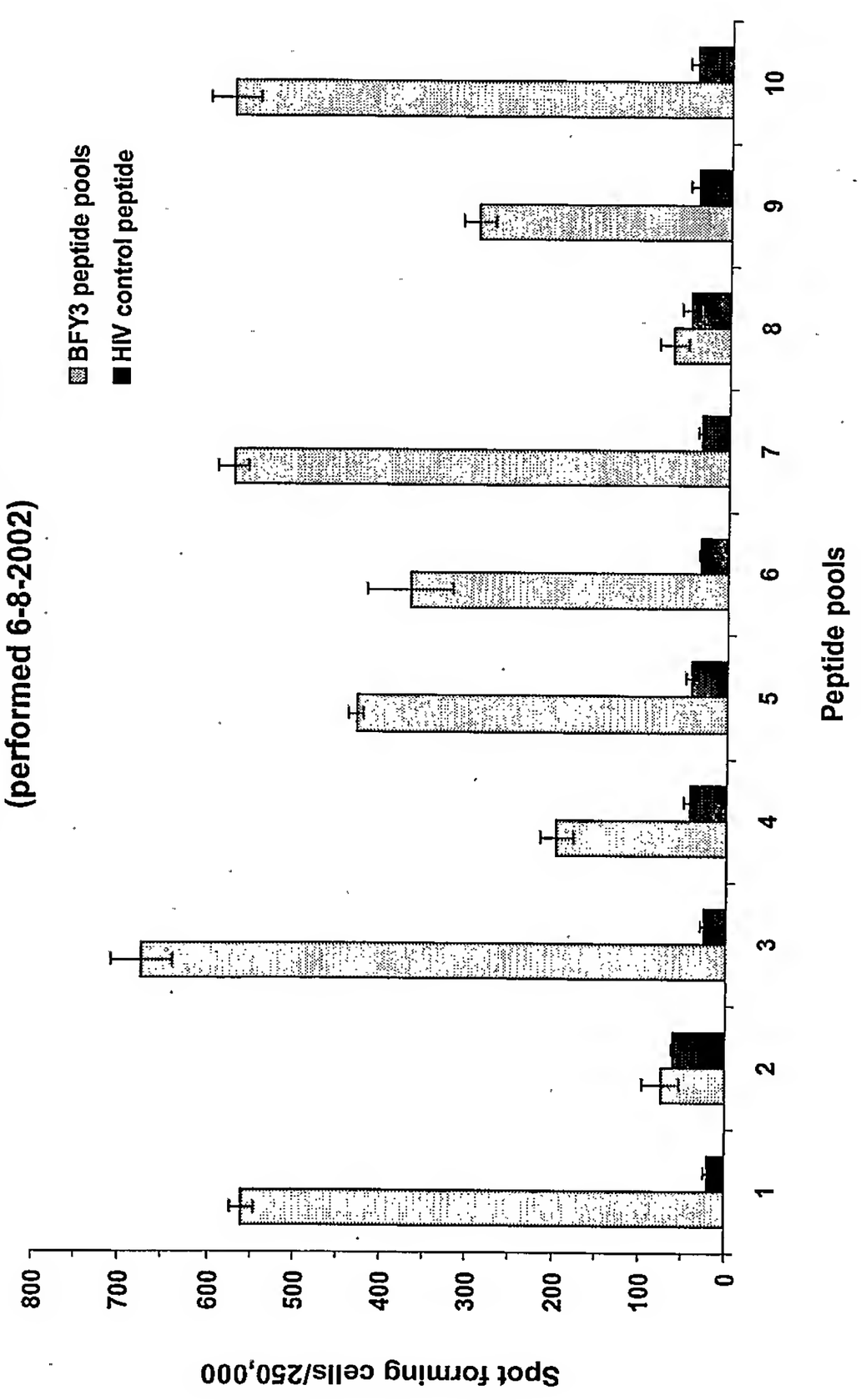

BFY3 IFN-γ ELISPOT after 4th stimulation
(performed 6-8-2002)
BFY3 peptide pools
HIV control peptide
Spot forming cells/250,000
0
100
200
300
400
500
600
700
800
1
2
3
4
5
6
7
8
9
10
Peptide pools

FIGURE 18B

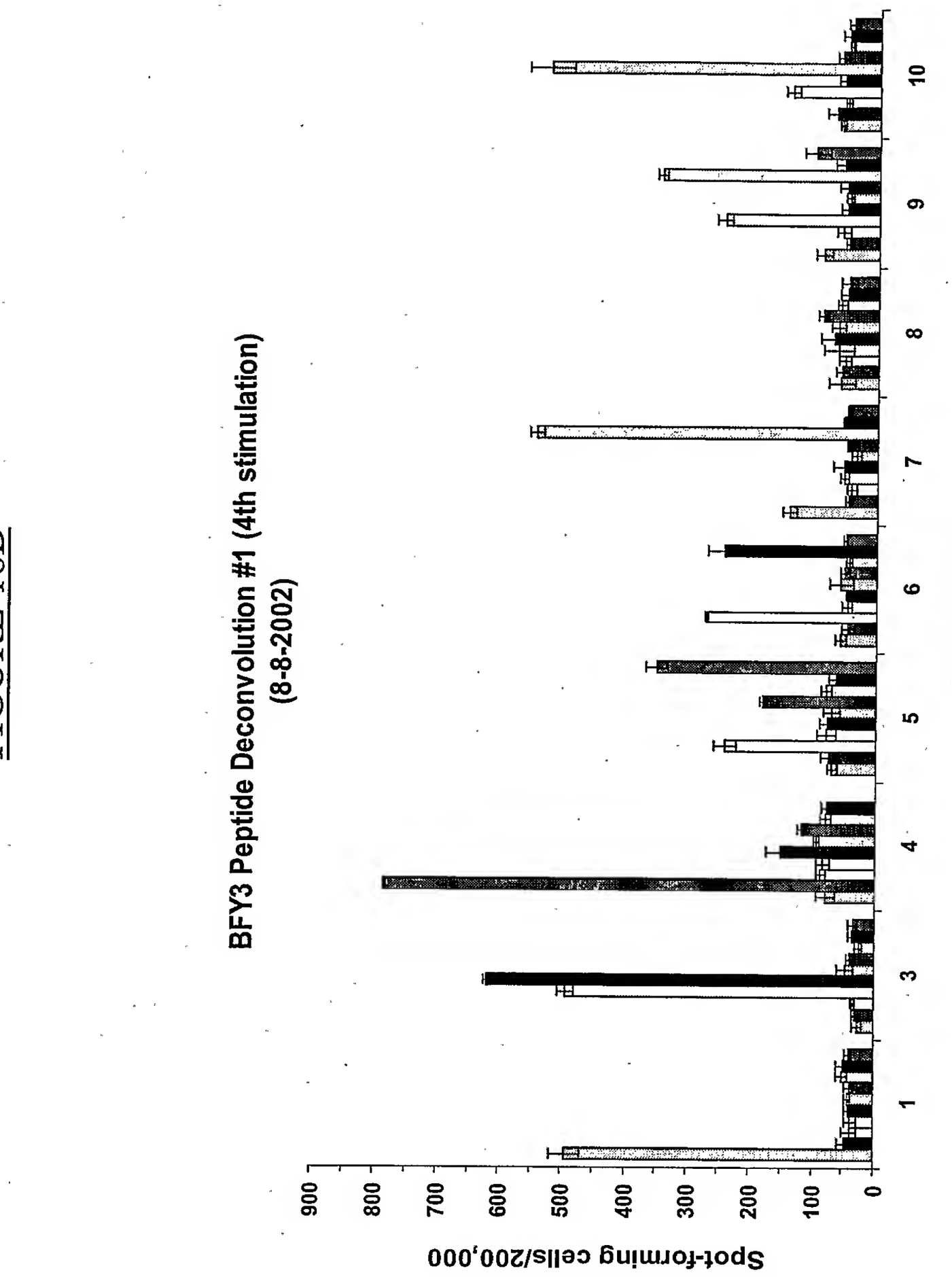

BFY3 Peptide Deconvolution #1 (4th stimulation)
(8-8-2002)
Spot-forming cells/200,000
0
100
200
300
400
500
600
700
800
900
1
3
4
5
6
7
8
9
10
Peptide group

FIGURE 18C

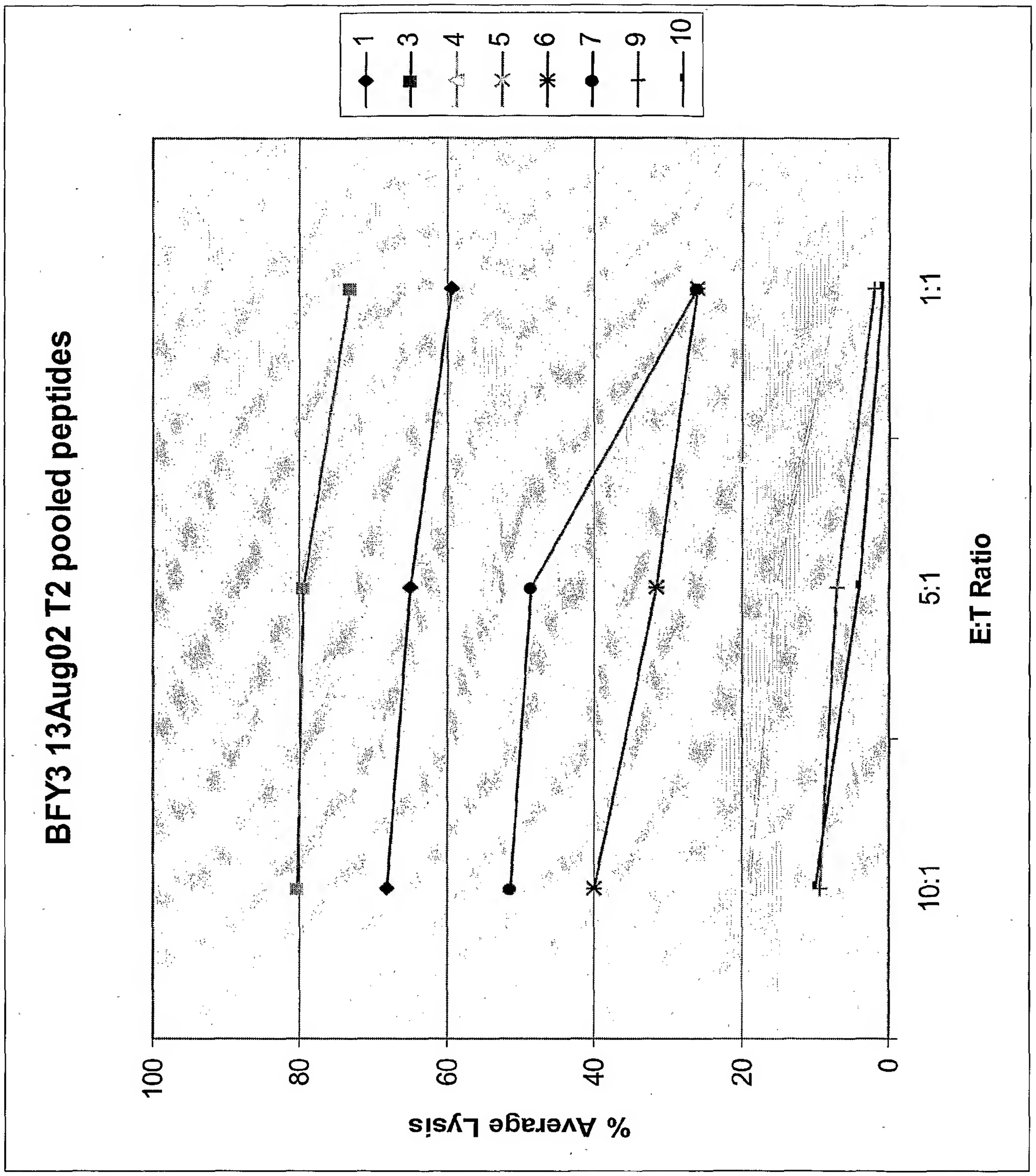

BFY3 13Aug02 T2 pooled peptides
1
3
4
5
6
7
9
10
% Average Lysis
100
80
60
40
20
0
E:T Ratio
10:1
5:1
1:1

FIGURE 18D

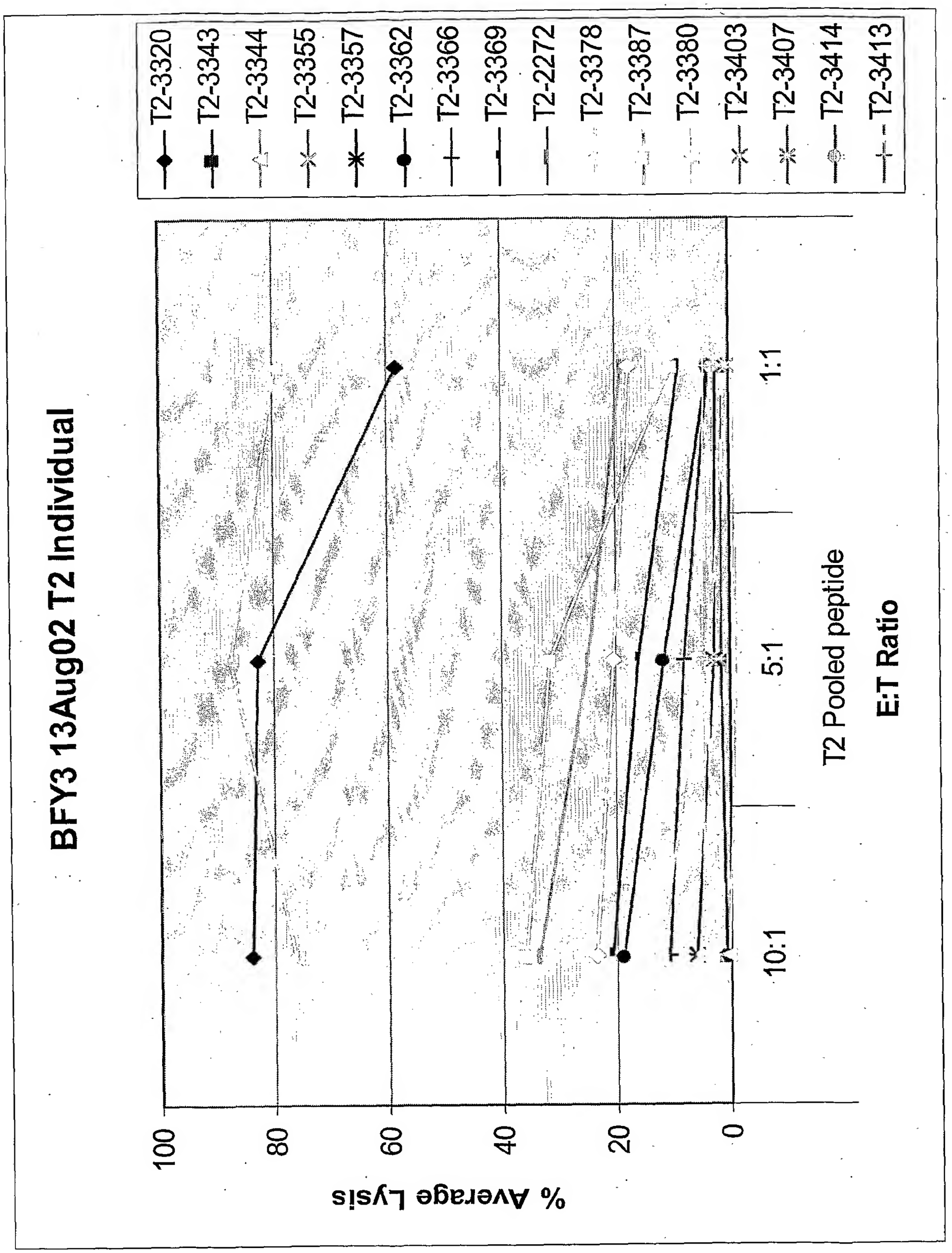
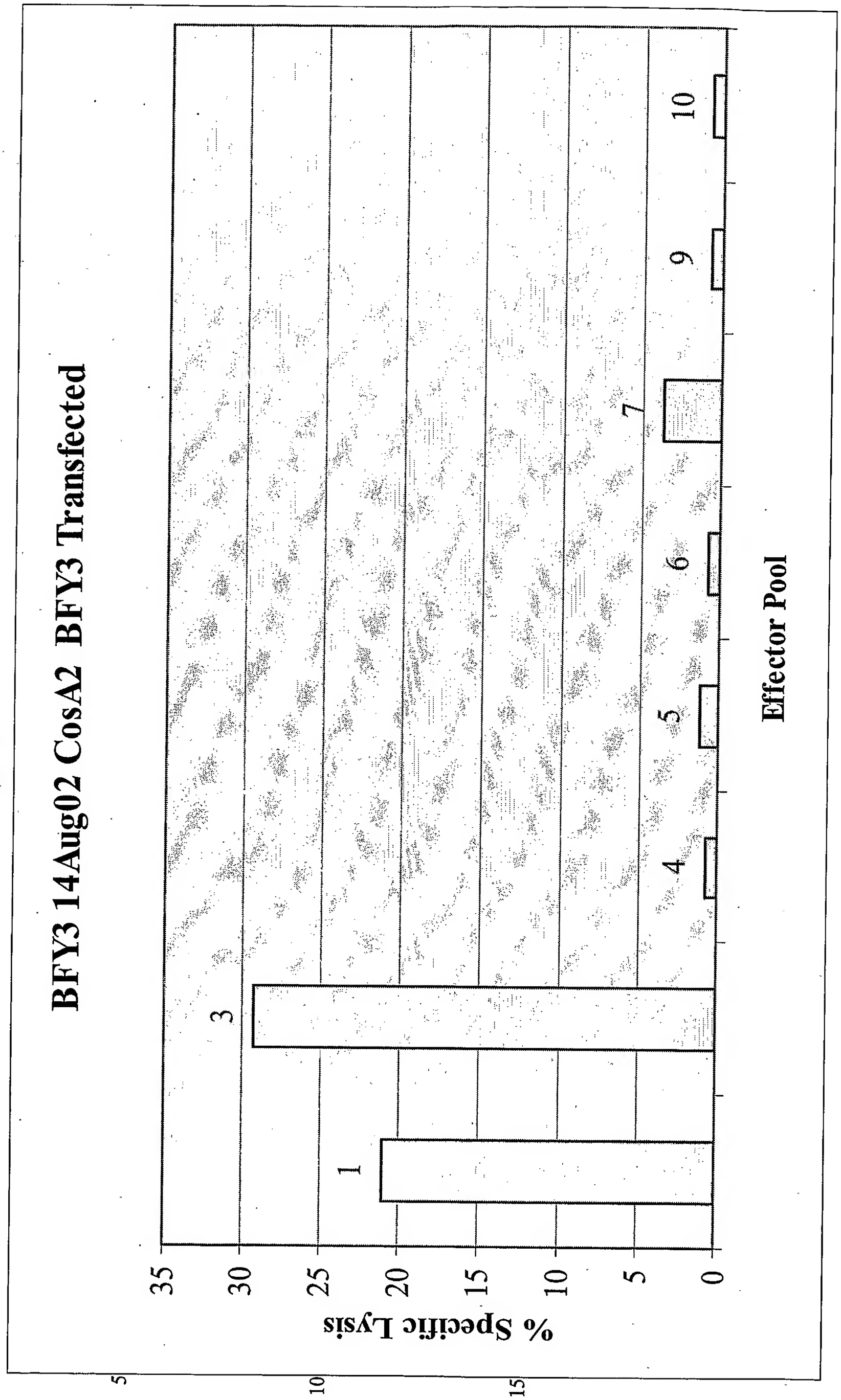

BFY3 13Aug02 T2 Individual
T2-3320
T2-3343
T2-3344
T2-3355
T2-3357
T2-3362
T2-3366
T2-3369
T2-2272
T2-3378
T2-3387
T2-3380
T2-3403
T2-3407
T2-3414
T2-3413
100
80
60
40
20
0
% Average Lysis
10:1
5:1
1:1
T2 Pooled peptide
E:T Ratio

FIGURE 18E


BFY3 14Aug02 CosA2 BFY3 Transfected
% Specific Lysis
0
5
10
15
20
25
30
35
Effector Pool
1
3
4
5
6
7
9
10

SEQUENCE LISTING

SEQ ID NO. 1: *AAC2-1 nucleotide sequence*

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TTGGCGGGCAACCCCTCAGAGCACACAAGGCAGTTCTCATCGCCTGCAGTGGCTTCTTCTATTCAATTTTCCGGGGC
CGTGCGGGAGTCGGGGTGGACGTGCTCTCTCTGCCCGGGGGTCCCGAAGCGAGAGGCTTCGCCCCTCTATTGGACTT
CATGTACACTTCGCGCCTGCGCCTCTCTCCAGCCACTGCACCAGCAGTCCTAGCGGCCGCCACCTATTTGCAGATGG
AGCACGTGGTCCAGGCATGCCACCGCTTCATCCAGGCCAGCTATGAACCTCTGGGCATCTCCCTGCGCCCCCTGGAA
GCAGAACCCCCAACACCCCCAACGGCCCCTCCACCAGGTAGTCCCAGGCGCTCCGAAGGACACCCAGACCCACCTAC
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AGTACATCGTGCTAAACTCTCAGGCCTCCCAAGCAGGGAGCCTGGTCGGGGAGAGAAGTTCTGGTCAACCTTGCCCC
CAAGCCAGGCTCCCCAGTGGAGACGAGGCCTCCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGTGAAGAAGGACC
CATTCCTGGTCCCCAGAGCAGGCTCTCTCCAACTGCTGCCACTGTGCAGTTCAAATGTGGGGCTCCAGCCAGTACCC
CCTACCTCCTCACATCCCAGGCTCAAGACACCTCTGGATCACCCTCTGAACGGGCTCGTCCACTACCGGGAGTGAAT
TTTTCAGCTGCCAGAACTGTGAGGCTGTGGCAGGGTGCTCATCGGGGGCTGGACTCCTTGGTTCCTGGGGACGAAGA
CAAACCCTATAAGTGTCAGCTGTGCCGGTCTTCGTTCCGCTACAAGGGCAACCTTGCCAGTCACCGTACAGTGCACA
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CGTGCTGATCCACACCGGGGAGAAGCCCTACCCTTGCCCTACCTGCGGAACCCGCTTCCGCCACCTGCAGACCCTCA
AGAGCCACGTTCGCATCCACACCGGAGAGAAGCCTTACCACTGCGACCCCTGTGGCCTGCATTTCCGGCACAAGAGT
CAACTGCGGCTGCATCTGCGCCAGAAACACGGAGCTGCTACCAACACCAAAGTGCACTACCACATTCTCGGGGGGCC
CTAGCTGAGCGCAGGCCCAGGCCCCACTTGCTTCCTGCGGGTGGGAAAGCTGCAGGCCCAGGCCTTGCTTCCCTATC
AGGCTTGGGCATAGGGGTGTGCCAGGCCACTTTGGTATCAGAAATTGCCACCCTCTTAATTTCTCACTGGGGAGAGC
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AGGAGAGAGCTAGCTAGGGGCTGGGAAAGGGGAGAGATTGGAGTCCTGGTCTCCCTAAGGGAATAGCCCTCCACCTG
TGGCCCCCATTGCATTCAGTTTATCTGTAAAATATAATTTATTGAGGCCTTTGGGTGGCACCGGGGCCTTCATTCGA
TTGCATTTCCCACTCCCCTCTTCCACAAGTGTGATTAAAAGTGACCAGAAACACAGAAGGTGAGATCACAGCTCTGC
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GTAATTCTAGGTCTGGAACCTTTATTTGTTCTAGGGCAGCTCTGGGAACATGCGGGATTGTGGAATTGGGTCAGGAA
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CTGCCCAGTCTCTATGAATGTTATGGCCTAGGGAAGAATCATGAAACTCTTTAGCTTGATTAGATGGTAAACAGTGT
TAACCCATCCTTTACTACAGAGGCATATGGGTTTGAATGTTACCTGGGGTTCTCTCTATTGAGTTGAGCCCCTTCTT
CCTTTAGTGGGTTTTGGACATCTTCTGGCAAGTGTCCAGATGCCAGAACCTTCTTTTCCTCTAGAAGGGATGGTGCT
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SEQ ID NO. 2: *AAC2-1 amino acid sequence*

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APPPGSPRRSEGHPDPPTESRSCSQGPPSPASPDPKACNWKKYKYIVLNSQASQAGSLVGERSSGQPCPQARLPSGD
EASSSSSSSSSSSEEGPIPGPQSRLSPTAATVQFKCGAPASTPYLLTSQAQDTSGSPSERARPLPGVNFSAARTVR
LWQGAHRGLDSLVPGDEDKPYKCQLCRSSFRYKGNLASHRTVHTGEKPYHCSICGARFNRPANLKTHSRIHSGEKPY
KCETCGSRFVQVAHLRAHVLIHTGEKPYPCPTCGTRFRHLQTLKSHVRIHTGEKPYHCDPCGLHFRHKSQLRLHLRQ
KHGAATNTKVHYHILGGP

SEQ ID NO. 3: *AAC2-2 nucleotide sequence*

TCTGCGTGTGCCGGGGCTAGGGGCTGGAAGTCCTGGCTCTAGTTGCACCTCGGAAGGAAAAGGCAAACAGAGGAGGGAAGGCGTCTT
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CAGGAACCCTCTCTGGTATTCTGGATGTTGTAGGTTCTCTAGCAGTCTAGAAATGGATACAGACATTTCTCTGTTCTTCAAGGGTGA
TAGGAACCATTATGTTGAGCCCAAAATGGAAGTAATAATAAATGCCTCCTGGAGGCTGTGGGTGTGGGGGATTCTGTATCTGGATTC
CGTATCACTCCAACTGGAGGCTGTGGGTGTGGGGGATTCTGTATCTGGATTCCGTATCACTCCAAGTGGAGGCTGGCAGGTTTTTCT
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TCCTTATCAGAGATGATGTGACCTTTTCTGACTCTGCCCAGTCTCTATGAATGTTATGGCCTAGGGAAGAATCATGAAACTCTTTAG
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TGAGCCCCTTCTTCCTTTAGTGGGTTTTGGACATCTTCTGGCAAGTGTCCAGATGCCAGAACCTTCTTTTCCTCTAGAAGGGATGGT
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GTGGGGTAGAACTTCAACAATAAGTCAGTTCTAGTGGCTGTCGCCTGGGGACTAGTGAGAAAGCTACTCTTCTCCCTCTTCCCTCTT
TCTCCCCATGGCCCCACTGCAGAATTAAAGAAGGAAGAAGGGAAGGCGGAGGAGTCTATAAGAAGGAATCATGATTTCTATTTAGCA
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CWRAAAAAAAAAAAAAAAAAAA

SEQ ID NO. 4: *AAC2-2 open reading frame*

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TGCAGTGGCTTCTTCTATTCAATTTTCCGGGGCCGTGCGGGAGTCGGGGTGGACGTGCTCTCTCTGCCCGGGGGTCCCGAAGCGAGA
GGCTTCGCCCCTCTATTGGACTTCATGTACACTTCGCGCCTGCGCCTCTCTCCAGCCACTGCACCAGCAGTCCTAGCGGCCGCCACC
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GAAGCAGAACCCCCAACACCCCCAACGGCCCCTCCACCAGGTAGTCCCAGGCGCTCCGAAGGACACCCAGACCCACCTACTGAATCT
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CGGGCTCGTCCACTACCGGGAAGTGAATTTTTCAGCTGCCAGAACTGTGAGGCTGTGGCAGGGTGCTCATCGGGGCTGGACTCCTTG
GTTCCTGGGGACGAAGACAAACCCTATAAGTGTCAGCTGTGCCGGTCTTCGTTCCGCTACAAGGGCAACCTTGCCAGTCATCGTACA
GTGCACACAGGGGAAAAGCCTTACCACTGCTCAATCTGCGGAGCCCGTTTTAACCGGCCAGCAAACCTGAAAACGCACAGCCGCATC
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ACCGGGGAGAAGCCCTACCCTTGCCCTACCTGCGGAACCCGCTTCCGCCACCTGCAGACCCTCAAGAGCCACGTTCGCATCCACACC
GGAGAGAAGCCTTACCACTGCGACCCCTGTGGCCTGCATTTCCGGCACAAGAGTCAACTGCGGCTGCATCTGCGCCAGAAACACGGA
GCTGCTACCAACACCAAAGTGCACTACCACATTCTCGGGGGGCCCTAG

SEQ ID NO. 5: *AAC2-2 amino acid sequence*

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RSCSQGPPSPASPDPKACNWKKYKYIVLNSQASQAGSLVGERSSGQPCPQARLPSGDEASSSSSSSSSSEEGPIPGPQSRLSPTAA
TVQFKCGAPASTPYLLTSQAQDTSGSPSERARPLPGSEFFSCQNCEAVAGCSSGLDSLVPGDEDKPYKCQLCRSSFRYKGNLASHRT
VHTGEKPYHCSICGARFNRPANLKTHSRIHSGEKPYKCETCGSRFVQVAHLRAHVLIHTGEKPYPCPTCGTRFRHLQTLKSHVRIHT
GEKPYHCDPCGLHFRHKSQLRLHLRQKHGAATNTKVHYHILGGP

SEQ ID NO. 6: *AAC2-2 FORWARD PRIMER*

CACCATGGGT TCCCCCGCCG CCCCGGA

SEQ ID NO. 7: *AAC2-2 REVERSE PRIMER*

CTAGGGCCCC CCGAGAATGT GGTAGTGCAC TTT

SEQ ID NO.: 3; *7524*
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SEQ ID NO.: 4; *7526*
GCCTGTGTCCCCTAGACTCCAACTCAGCAACGGAAATAGAACTCTGACCCTGTTTAACGTGACCAGGAAC

SEQ ID NO.: 5; *7528*
ACGTGCTTTACGGACCCGATGCTCCTACAATCAGCCCTCTAAACACAAGCTATAGATCAGGGGAAAATCT

SEQ ID NO.: 6; *7533*
ACGTTAAACAGGGTCAGAGTTCTATTTCCGTTGCTGAGTTGGAGTCTAGGGGACACAGGCAGGGACTGGT

SEQ ID NO.: 7; *7535*
CTGATCTATAGCTTGTGTTTAGAGGGCTGATTGTAGGAGCATCGGGTCCGTAAAGCACGTTGAGAATCAC

SEQ ID NO.: 8; *7537*
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SEQ ID NO.: 9; *7567*
GACACAGCAAGCTACAAATGCGAAACCCAAAATCCAGTCAGCGCCAGGAGGTCTGATTCAGTGATTCTCA

SEQ ID NO.: 10; *7568*
TGAATCAGACCTCCTGGCGCTGACTGGATTTTGGGTTTCGCATTTGTAGCTTGCTGTGTCGTTCCTGGTC

SEQ ID NO.: 11; *7576*

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A

SEQ ID NO.: 12; *7587*
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SEQ ID NO.: 13; *7677*
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SEQ ID NO.: 14; *7678*
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SEQ ID NO.: 16; *7680*
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CC

SEQ ID NO.: 17; *7681*
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SEQ ID NO.: 19; *7683*
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CA

SEQ ID NO.: 20; *7684*
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TC

SEQ ID NO.: 21; *7685*
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TG

SEQ ID NO.: 22; *7686*
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SEQ ID NO.: 23: *BFA4 cDNA*
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ACAGCAAAATCTTGGCCCTTCATAACATGGTGCAGTTCAGCCATTCCAAAGACTTCCAGAAGGTCAACCGTTCTGTGTTTTCTGGTG
TGCTGCAGGACATCAATTCTTCAAGGCCTGTTTTACTAAATGGGACCTATGATGTGCAGGTGACTTCAGGTGGAACATTCATTGGCA
TTGGACGGAAAACACCAGATTGCCAAGGGAACACCAAGTATTTCCGCTGTAAATTCTGCAATTTCACTTATATGGGCAACTCATCCA

CCGAATTAGAACAACATTTTCTTCAGACTCACCCAAACAAAATAAAAGCTTCTCTCCCCTCCTCTGAGGTTGCAAAACCTTCAGAGA
AAAACTCTAACAAGTCCATCCCTGCACTTCAATCCAGTGATTCTGGAGACTTGGGAAAATGGCAGGACAAGATAACAGTCAAAGCAG
GAGATGACACTCCTGTTGGGTACTCAGTGCCCATAAAGCCCCTCGATTCCTCTAGACAAAATGGTACAGAGGCCACCAGTTACTACT
GGTGTAAATTTTGTAGTTTCAGCTGTGAGTCATCTAGCTCACTTAAACTGCTAGAACATTATGGCAAGCAGCACGGAGCAGTGCAGT
CAGGCGGCCTTAATCCAGAGTTAAATGATAAGCTTTCCAGGGGCTCTGTCATTAATCAGAATGATCTAGCCAAAAGTTCAGAAGGAG
AGACAATGACCAAGACAGACAAGAGCTCGAGTGGGGCTAAAAAGAAGGACTTCTCCAGCAAGGGAGCCGAGGATAATATGGTAACGA
GCTATAATTGTCAGTTCTGTGACTTCCGATATTCCAAAAGCCATGGCCCTGATGTAATTGTAGTGGGGCCACTTCTCCGTCATTATC
AACAGCTCCATAACATTCACAAGTGTACCATTAAACACTGTCCATTCTGTCCCAGAGGACTTTGCAGCCCAGAAAAGCACCTTGGAG
AAATTACTTATCCGTTTGCTTGTAGAAAAAGTAATTGTTCCCACTGTGCACTCTTGCTTCTGCACTTGTCTCCTGGGGCGGCTGGAA
GCTCGCGAGTCAAACATCAGTGCCATCAGTGTTCATTCACCACCCCTGACGTAGATGTACTCCTCTTTCACTATGAAAGTGTGCATG
AGTCCCAAGCATCGGATGTCAAACAAGAAGCAAATCACCTGCAAGGATCGGATGGGCAGCAGTCTGTCAAGGAAAGCAAAGAACACT
CATGTACCAAATGTGATTTTATTACCCAAGTGGAAGAAGAGATTTCCCGACACTACAGGAGAGCACACAGCTGCTACAAATGCCGTC
AGTGCAGTTTTACAGCTGCCGATACTCAGTCACTACTGGAGCACTTCAACACTGTTCACTGCCAGGAACAGGACATCACTACAGCCA
ACGGCGAAGAGGACGGTCATGCCATATCCACCATCAAAGAGGAGCCCAAAATTGACTTCAGGGTCTACAATCTGCTAACTCCAGACT
CTAAAATGGGAGAGCCAGTTTCTGAGAGTGTGGTGAAGAGAGAAGCTGGAAGAGAAGGACGGGCTCAAAGAGAAAGTTTGGACCG
AGAGTTCCAGTGATGACCTTCGCAATGTGACTTGGAGAGGGGCAGACATCCTGCGGGGGAGTCCGTCATACACCCAAGCAAGCCTGG
GGCTGCTGACGCCTGTGTCTGGCACCCAAGAGCAGACAAAGACTCTAAGGGATAGTCCCAATGTGGAGGCCGCCCATCTGGCGCGAC
CTATTTATGGCTTGGCTGTGGAAACCAAGGGATTCCTGCAGGGGGCGCCAGCTGGCGGAGAGAAGTCTGGGGCCCTCCCCCAGCAGT
ATCCTGCATCGGGAGAAAACAAGTCCAAGGATGAATCCCAGTCCCTGTTACGGAGGCGTAGAGGCTCCGGTGTTTTTTGTGCCAATT
GCCTGACCACAAAGACCTCTCTCTGGCGAAAGAATGCAAATGGCGGATATGTATGCAACGCGTGTGGCCTCTACCAGAAGCTTCACT
CGACTCCCAGGCCTTTAAACATCATTAAACAAAACAACGGTGAGCAGATTATTAGGAGGAGAACAAGAAAGCGCCTTAACCCAGAGG
CACTTCAGGCTGAGCAGCTCAACAAACAGCAGAGGGGCAGCAATGAGGAGCAAGTCAATGGAAGCCCGTTAGAGAGGAGGTCAGAAG
ATCATCTAACTGAAAGTCACCAGAGAGAAATTCCACTCCCCAGCCTAAGTAAATACGAAGCCCAGGGTTCATTGACTAAAAGCCATT
CTGCTCAGCAGCCAGTCCTGGTCAGCCAAACTCTGGATATTCACAAAAGGATGCAACCTTTGCACATTCAGATAAAAAGTCCTCAGG
AAAGTACTGGAGATCCAGGAAATAGTTCATCCGTATCTGAAGGGAAAGGAAGTTCTGAGAGAGGCAGTCCTATAGAAAAGTACATGA
GACCTGCGAAACACCCAAATTATTCACCACCAGGCAGCCCTATTGAAAAGTACCAGTACCCACTTTTTGGACTTCCCTTTGTACATA
ATGACTTCCAGAGTGAAGCTGATTGGCTGCGGTTCTGGAGTAAATATAAGCTCTCCGTTCCTGGGAATCCGCACTACTTGAGTCACG
TGCCTGGCCTACCAAATCCTTGCCAAAACTATGTGCCTTATCCCACCTTCAATCTGCCTCCTCATTTTTCAGCTGTTGGATCAGACA
ATGACATTCCTCTAGATTTGGCGATCAAGCATTCCAGACCTGGGCCAACTGCAAACGGTGCCTCCAAGGAGAAAACGAAGGCACCAC
CAAATGTAAAAAATGAAGGTCCCTTGAATGTAGTAAAAACAGAGAAAGTTGATAGAAGTACTCAAGATGAACTTTCAACAAAATGTG
TGCACTGTGGCATTGTCTTTCTGGATGAAGTGATGTATGCTTTGCATATGAGTTGCCATGGTGACAGTGGACCTTTCCAGTGCAGCA
TATGCCAGCATCTTTGCACGGACAAATATGACTTCACAACACATATCCAGAGGGGCCTGCATAGGAACAATGCACAAGTGGAAAAAA
ATGGAAAACCTAAAGAGTAA

SEQ ID NO.: 24: BFA4 Amino Acid Sequence

MVRKKNPPLRNVASEGEGQILEPIGTESKVSGKNKEFSADQMSENTDQSDAAELNHKEEHSLHVQDPSSS
SKKDLKSAVLSEKAGFNYESPSKGGNFPSFPHDEVTDRNMLAFSFPAAGGVCEPLKSPQRAEADDPQDMA
CTPSGDSLETKEDQKMSPKATEETGQAQSGQANCQGLSPVSVASKNPQVPSDGGVRLNKSKTDLLVNDNP
DPAPLSPELQDFKCNICGYGYYGNDPTDLIKHFRKYHLGLHNRTRQDAELDSKILALHNMVQFSHSKDFQ
KVNRSVFSGVLQDINSSRPVLLNGTYDVQVTSGGTFIGIGRKTPDCQGNTKYFRCKFCNFTYMGNSSTEL
EQHFLQTHPNKIKASLPSSEVAKPSEKNSNKSIPALQSSDSGDLGKWQDKITVKAGDDTPVGYSVPIKPL
DSSRQNGTEATSYYWCKFCSFSCESSSSLKLLEHYGKQHGAVQSGGLNPELNDKLSRGSVINQNDLAKSS
EGETMTKTDKSSSGAKKKDFSSKGAEDNMVTSYNCQFCDFRYSKSHGPDVIVVGPLLRHYQQLHNIHKCT
IKHCPFCPRGLCSPEKHLGEITYPFACRKSNCSHCALLLLHLSPGAAGSSRVKHQCHQCSFTTPDVDVLL
FHYESVHESQASDVKQEANHLQGSDGQQSVKESKEHSCTKCDFITQVEEEISRHYRRAHSCYKCRQCSFT
AADTQSLLEHFNTVHCQEQDITTANGEEDGHAISTIKEEPKIDFRVYNLLTPDSKMGEPVSESVVKREKL
EEKDGLKEKVWTESSSDDLRNVTWRGADILRGSPSYTQASLGLLTPVSGTQEQTKTLRDSPNVEAAHLAR
PIYGLAVETKGFLQGAPAGGEKSGALPQQYPASGENKSKDESQSLLRRRRGSGVFCANCLTTKTSLWRKN
ANGGYVCNACGLYQKLHSTPRPLNIIKQNNGEQIIRRRTRKRLNPEALQAEQLNKQQRGSNEEQVNGSPL
ERRSEDHLTESHQREIPLPSLSKYEAQGSLTKSHSAQQPVLVSQTLDIHKRMQPLHIQIKSPQESTGDPG
NSSSVSEGKGSSERGSPIEKYMRPAKHPNYSPPGSPIEKYQYPLFGLPFVHNDFQSEADWLRFWSKYKLS
VPGNPHYLSHVPGLPNPCQNYVPYPTFNLPPHFSAVGSDNDIPLDLAIKHSRPGPTANGASKEKTKAPPN
VKNEGPLNVVKTEKVDRSTQDELSTKCVHCGIVFLDEVMYALHMSCHGDSGPFQCSICQHLCTDKYDFTT
HIQRGLHRNNAQVEKNGKPKE

SEQ ID NO.: 25: *BCY1 cDNA Sequence*

ATGGCCGAGCTGCGCCTGAAGGGCAGCAGCAACACCACGGAGTGTGTTCCCGTGCCCACCTCCGAGCACGTGGCCGAG
ATCGTGGGCAGGCAAGGCTGCAAGATTAAGGCCTTGAGGGCCAAGACCAACACCTACATCAAGACACCGGTGAGGGGC
GAGGAACCAGTGTTCATGGTGACAGGGCGACGGGAGGACGTGGCCACAGCCCGGCGGGAAATCATCTCAGCAGCGGAG
CACTTCTCCATGATCCGTGCCTCCCGCAACAAGTCAGGCGCCGCCTTTGGTGTGGCTCCTGCTCTGCCCGGCCAGGTG
ACCATCCGTGTGCGGGTGCCCTACCGCGTGGTGGGGCTGGTGGTGGGCCCCAAAGGGGCAACCATCAAGCGCATCCAG
CAGCAAACCAACACATACATTATCACACCAAGCCGTGACCGCGACCCCGTGTTCGAGATCACGGGTGCCCCAGGCAAC
GTGGAGCGTGCGCGCGAGGAGATCGAGACGCACATCGCGGTGCGCACTGGCAAGATCCTCGAGTACAACAATGAAAAC
GACTTCCTGGCGGGGAGCCCCGACGCAGCAATCGATAGCCGCTACTCCGACGCCTGGCGGGTGCACCAGCCCGGCTGC
AAGCCCCTCTCCACCTTCCGGCAGAACAGCCTGGGCTGCATCGGCGAGTGCGGAGTGGACTCTGGCTTTGAGGCCCCA
CGCCTGGGTGAGCAGGGCGGGGACTTTGGCTACGGCGGGTACCTCTTTCCGGGCTATGGCGTGGGCAAGCAGGATGTG
TACTACGGCGTGGCCGAGACTAGCCCCCCGCTGTGGGCGGGCCAGGAGAACGCCACGCCCACCTCCGTGCTCTTCTCC
TCTGCCTCCTCCTCCTCCTCCTCTTCCGCCAAGGCCCGCGCTGGGCCCCCGGGCGCACACCGCTCCCCTGCCACTTCC
GCGGGACCCGAGCTGGCCGGACTCCCGAGGCGCCCCCCGGGAGAGCCGCTCCAGGGCTTCTCTAAACTTGGTGGGGGC
GGCCTGCGGAGCCCCGGCGGCGGGCGGGATTGCATGGTCTGCTTTGAGAGCGAAGTGACTGCCGCCCTTGTGCCCTGC
GGACACAACCTGTTCTGCATGGAGTGTGCAGTACGCATCTGCGAGAGGACGGACCCAGAGTGTCCCGTCTGCCACATC
ACAGCCGCGCAAGCCATCCGAATATTCTCCTAA

SEQ ID NO.: 26: *BCY1 Amino Acid Sequence*

MAELRLKGSS NTTECVPVPT SEHVAEIVGR QGCKIKALRA KTNTYIKTPV RGEEPVFMVT GRREDVATAR
REIISAAEHF SMIRASRNKS GAAFGVAPAL PGQVTIRVRV PYRVVGLVVG PKGATIKRIQ QQTNTYIITP
SRDRDPVFEI TGAPGNVERA REEIETHIAV RTGKILEYNN ENDFLAGSPD AAIDSRYSDA WRVHQPGCKP
LSTFRQNSLG CIGECGVDSG FEAPRLGEQG GDFGYGGYLF PGYGVGKQDV YYGVAETSPP LWAGQENATP
TSVLFSSASS SSSSSAKARA GPPGAHRSPA TSAGPELAGL PRRPPGEPLQ GFSKLGGGGL RSPGGGRDCM
VCFESEVTAA LVPCGHNLFC MECAVRICER TDPECPVCHI TAAQAIRIFS

SEQ ID NO.: 27: *BFA5 Nucleotide Sequence*

ATGACAAAGAGGAAGAAGACCATCAACCTTAATATACAAGACGCCCAGAAGAGGACTGCTCTACACTGGGC
CTGTGTCAATGGCCATGAGGAAGTAGTAACATTTCTGGTAGACAGAAAGTGCCAGCTTGACGTCCTTGATG
GCGAACACAGGACACCTCTGATGAAGGCTCTACAATGCCATCAGGAGGCTTGTGCAAATATTCTGATAGAT
TCTGGTGCCGATATAAATCTCGTAGATGTGTATGGCAACATGGCTCTCCATTATGCTGTTTATAGTGAGAT
TTTGTCAGTGGTGGCAAAACTGCTGTCCCATGGTGCAGTCATCGAAGTGCACAACAAGGCTAGCCTCACAC
CACTTTTACTATCCATAACGAAAAGAAGTGAGCAAATTGTGGAATTTTTGCTGATAAAAAATGCAAATGCG

AATGCAGTTAATAAGTATAAATGCACAGCCCTCATGCTTGCTGTATGTCATGGATCATCAGAGATAGTTGG
CATGCTTCTTCAGCAAAATGTTGACGTCTTTGCTGCAGATATATGTGGAGTAACTGCAGAACATTATGCTG
TTACTTGTGGATTTCATCACATTCATGAACAAATTATGGAATATATACGAAAATTATCTAAAAATCATCAA
AATACCAATCCAGAAGGAACATCTGCAGGAACACCTGATGAGGCTGCACCCTTGGCGGAAAGAACACCTGA
CACAGCTGAAAGCTTGGTGGAAAAAACACCTGATGAGGCTGCACCCTTGGTGGAAAGAACACCTGACACGG
CTGAAAGCTTGGTGGAAAAAACACCTGATGAGGCTGCATCCTTGGTGGAGGGAACATCTGACAAAATTCAA
TGTTTGGAGAAAGCGACATCTGGAAAGTTCGAACAGTCAGCAGAAGAAACACCTAGGGAAATTACGAGTCC
TGCAAAAGAAACATCTGAGAAATTTACGTGGCCAGCAAAAGGAAGACCTAGGAAGATCGCATGGGAGAAAA
AAGAAGACACACCTAGGGAAATTATGAGTCCCGCAAAAGAAACATCTGAGAAATTTACGTGGGCAGCAAAA
GGAAGACCTAGGAAGATCGCATGGGAGAAAAAAGAAACACCTGTAAAGACTGGATGCGTGGCAAGAGTAAC
ATCTAATAAAACTAAAGTTTTGGAAAAAGGAAGATCTAAGATGATTGCATGTCCTACAAAAGAATCATCTA
CAAAAGCAAGTGCCAATGATCAGAGGTTCCCATCAGAATCCAAACAAGAGGAAGATGAAGAATATTCTTGT
GATTCTCGGAGTCTCTTTGAGAGTTCTGCAAAGATTCAAGTGTGTATACCTGAGTCTATATATCAAAAAGT
AATGGAGATAAATAGAGAAGTAGAAGAGCCTCCTAAGAAGCCATCTGCCTTCAAGCCTGCCATTGAAATGC
AAAACTCTGTTCCAAATAAAGCCTTTGAATTGAAGAATGAACAAACATTGAGAGCAGATCCGATGTTCCCA
CCAGAATCCAAACAAAAGGACTATGAAGAAAATTCTTGGGATTCTGAGAGTCTCTGTGAGACTGTTTCACA
GAAGGATGTGTGTTTACCCAAGGCTACACATCAAAAAGAAATAGATAAAATAAATGGAAAATTAGAAGAGT
CTCCTAATAAAGATGGTCTTCTGAAGGCTACCTGCGGAATGAAAGTTTCTATTCCAACTAAAGCCTTAGAA
TTGAAGGACATGCAAACTTTCAAAGCGGAGCCTCCGGGGAAGCCATCTGCCTTCGAGCCTGCCACTGAAAT
GCAAAAGTCTGTCCCAAATAAAGCCTTGGAATTGAAAAATGAACAAACATGGAGAGCAGATGAGATACTCC
CATCAGAATCCAAACAAAAGGACTATGAAGAAAATTCTTGGGATACTGAGAGTCTCTGTGAGACTGTTTCA
CAGAAGGATGTGTGTTTACCCAAGGCTGCGCATCAAAAAGAAATAGATAAAATAAATGGAAAATTAGAAGG
GTCTCCTGTTAAAGATGGTCTTCTGAAGGCTAACTGCGGAATGAAAGTTTCTATTCCAACTAAAGCCTTAG
AATTGATGGACATGCAAACTTTCAAAGCAGAGCCTCCCGAGAAGCCATCTGCCTTCGAGCCTGCCATTGAA
ATGCAAAAGTCTGTTCCAAATAAAGCCTTGGAATTGAAGAATGAACAAACATTGAGAGCAGATGAGATACT
CCCATCAGAATCCAAACAAAAGGACTATGAAGAAAGTTCTTGGGATTCTGAGAGTCTCTGTGAGACTGTTT
CACAGAAGGATGTGTGTTTACCCAAGGCTACACATCAAAAAGAAATAGATAAAATAAATGGAAAATTAGAA
GAGTCTCCTGATAATGATGGTTTTCTGAAGGCTCCCTGCAGAATGAAAGTTTCTATTCCAACTAAAGCCTT
AGAATTGATGGACATGCAAACTTTCAAAGCAGAGCCTCCCGAGAAGCCATCTGCCTTCGAGCCTGCCATTG
AAATGCAAAAGTCTGTTCCAAATAAAGCCTTGGAATTGAAGAATGAACAAACATTGAGAGCAGATCAGATG
TTCCCTTCAGAATCAAAACAAAAGAAGGTTGAAGAAAATTCTTGGGATTCTGAGAGTCTCCGTGAGACTGT
TTCACAGAAGGATGTGTGTGTACCCAAGGCTACACATCAAAAAGAAATGGATAAAATAAGTGGAAAATTAG
AAGATTCAACTAGCCTATCAAAAATCTTGGATACAGTTCATTCTTGTGAAAGAGCAAGGGAACTTCAAAAA
GATCACTGTGAACAACGTACAGGAAAAATGGAACAAATGAAAAAGAAGTTTTGTGTACTGAAAAAGAAACT
GTCAGAAGCAAAAGAAATAAAATCACAGTTAGAGAACCAAAAAGTTAAATGGGAACAAGAGCTCTGCAGTG
TGAGATTGACTTTAAACCAAGAAGAAGAGAAGAGAAGAAATGCCGATATATTAAATGAAAAAATTAGGGAA
GAATTAGGAAGAATCGAAGAGCAGCATAGGAAAGAGTTAGAAGTGAAACAACAACTTGAACAGGCTCTCAG
AATACAAGATATAGAATTGAAGAGTGTAGAAAGTAATTTGAATCAGGTTTCTCACACTCATGAAAATGAAA
ATTATCTCTTACATGAAAATTGCATGTTGAAAAAGGAAATTGCCATGCTAAAACTGGAAATAGCCACACTG
AAACACCAATACCAGGAAAAGGAAAATAAATACTTTGAGGACATTAAGATTTTAAAAGAAAAGAATGCTGA
ACTTCAGATGACCCTAAAACTGAAAGAGGAATCATTAACTAAAAGGGCATCTCAATATAGTGGGCAGCTTA
AAGTTCTGATAGCTGAGAACACAATGCTCACTTCTAAATTGAAGGAAAAACAAGACAAAGAAATACTAGAG
GCAGAAATTGAATCACACCATCCTAGACTGGCTTCTGCTGTACAAGACCATGATCAAATTGTGACATCAAG
AAAAAGTCAAGAACCTGCTTTCCACATTGCAGGAGATGCTTGTTTGCAAAGAAAAATGAATGTTGATGTGA
GTAGTACGATATATAACAATGAGGTGCTCCATCAACCACTTTCTGAAGCTCAAAGGAAATCCAAAAGCCTA
AAAATTAATCTCAATTATGCAGGAGATGCTCTAAGAGAAAATACATTGGTTTCAGAACATGCACAAAGAGA
CCAACGTGAAACACAGTGTCAAATGAAGGAAGCTGAACACATGTATCAAAACGAACAAGATAATGTGAACA
AACACACTGAACAGCAGGAGTCTCTAGATCAGAAATTATTTCAACTACAAAGCAAAAAATATGTGGCTTCAA
CAGCAATTAGTTCATGCACATAAGAAAGCTGACAACAAAAGCAAGATAACAATTGATATTCATTTTCTTGA
GAGGAAAATGCAACATCATCTCCTAAAAGAGAAAAATGAGGAGATATTTAATTACAATAACCATTTAAAAA
ACCGTATATATCAATATGAAAAAGAGAAAGCAGAAACAGAAAACTCATGA

SEQ ID NO.: 28: *BFA5 Amino Acid Sequence*

MTKRKKTINLNIQDAQKRTALHWACVNGHEEVVTFLVDRKCQLDVLDGEHRTPLMKALQCHQEACANILIDSGADINL
VDVYGNMALHYAVYSEILSVVAKLLSHGAVIEVHNKASLTPLLLSITKRSEQIVEFLLIKNANANAVNKYKCTALMLA
VCHGSSEIVGMLLQQNVDVFAADICGVTAEHYAVTCGFHHIHEQIMEYIRKLSKNHQNTNPEGTSAGTPDEAAPLAER
TPDTAESLVEKTPDEAAPLVERTPDTAESLVEKTPDEAASLVEGTSDKIQCLEKATSGKFEQSAEETPREITSPAKET
SEKFTWPAKGRPRKIAWEKKEDTPREIMSPAKETSEKFTWAAKGRPRKIAWEKKETPVKTGCVARVTSNKTKVLEKGR
SKMIACPTKESSTKASANDQRFPSESKQEEDEEYSCDSRSLFESSAKIQVCIPESIYQKVMEINREVEEPPKKPSAFK
PAIEMQNSVPNKAFELKNEQTLRADPMFPPESKQKDYEENSWDSESLCETVSQKDVCLPKATHQKEIDKINGKLEESP
NKDGLLKATCGMKVSIPTKALELKDMQTFKAEPPGKPSAFEPATEMQKSVPNKALELKNEQTWRADEILPSESKQKDY
EENSWDTESLCETVSQKDVCLPKAAHQKEIDKINGKLEGSPVKDGLLKANCGMKVSIPTKALELMDMQTFKAEPPEKP
SAFEPAIEMQKSVPNKALELKNEQTLRADEILPSESKQKDYEESSWDSESLCETVSQKDVCLPKATHQKEIDKINGKL
EESPDNDGFLKAPCRMKVSIPTKALELMDMQTFKAEPPEKPSAFEPAIEMQKSVPNKALELKNEQTLRADQMFPSESK
QKKVEENSWDSESLRETVSQKDVCVPKATHQKEMDKISGKLEDSTSLSKILDTVHSCERARELQKDHCEQRTGKMEQM
KKKFCVLKKKLSEAKEIKSQLENQKVKWEQELCSVRLTLNQEEEKRRNADILNEKIREELGRIEEQHRKELEVKQQLE
QALRIQDIELKSVESNLNQVSHTHENENYLLHENCMLKKEIAMLKLEIATLKHQYQEKENKYFEDIKILKEKNAELQM
TLKLKEESLTKRASQYSGQLKVLIAENTMLTSKLKEKQDKEILEAEIESHHPRLASAVQDHDQIVTSRKSQEPAFHIA
GDACLQRKMNVDVSSTIYNNEVLHQPLSEAQRKSKSLKINLNYAGDALRENTLVSEHAQRDQRETQCQMKEAEHMYQN
EQDNVNKHTEQQESLDQKLFQLQSKNMWLQQQLVHAHKKADNKSKITIDIHFLERKMQHHLLKEKNEEIFNYNNHLKN
RIYQYEKEKAETENS

SEQ ID NO. 29: *BCZ4 Nucleotide Sequence*

ATGGACATTGAAGCATATCTTGAAAGAATTGGCTATAAGAAGTCTAGGAACAAATTGGACTTGGAAACATTAACTGAC
ATTCTTCAACACCAGATCCGAGCTGTTCCCTTTGAGAACCTTAACATCCATTGTGGGGATGCCATGGACTTAGGCTTA
GAGGCCATTTTTGATCAAGTTGTGAGAAGAAATCGGGGTGGATGGTGTCTCCAGGTCAATCATCTTCTGTACTGGGCT
CTGACCACTATTGGTTTTGAGACCACGATGTTGGGAGGGTATGTTTACAGCACTCCAGCCAAAAAATACAGCACTGGC
ATGATTCACCTTCTCCTGCAGGTGACCATTGATGGCAGGAACTACATTGTCGATGCTGGGTTTGGACGCTCATACCAG
ATGTGGCAGCCTCTGGAGTTAATTTCTGGGAAGGATCAGCCTCAGGTGCCTTGTGTCTTCCGTTTGACGGAAGAGAAT
GGATTCTGGTATCTAGACCAAATCAGAAGGGAACAGTACATTCCAAATGAAGAATTTCTTCATTCTGATCTCCTAGAA
GACAGCAAATACCGAAAAATCTACTCCTTTACTCTTAAGCCTCGAACAATTGAAGATTTTGAGTCTATGAATACATAC
CTGCAGACATCTCCATCATCTGTGTTTACTAGTAAATCATTTTGTTCCTTGCAGACCCCAGATGGGGTTCACTGTTTG
GTGGGCTTCACCCTCACCCATAGGAGATTCAATTATAAGGACAATACAGATCTAATAGAGTTCAAGACTCTGAGTGAG
GAAGAAATAGAAAAAGTGCTGAAAAATATATTTAATATTTCCTTGCAGAGAAAGCTTGTGCCCAAACATGGTGATAGA
TTTTTTACTATTTAG

SEQ ID NO. 30: *BCZ4 Amino Acid Sequence*

MDIEAYLERIGYKKSRNKLDLETLTDILQHQIRAVPFENLNIHCGDAMDLGLEAIFDQVVRRNRGGWCLQVNHLLYWA
LTTIGFETTMLGGYVYSTPAKKYSTGMIHLLLQVTIDGRNYIVDAGFGRSYQMWQPLELISGKDQPQVPCVFRLTEEN
GFWYLDQIRREQYIPNEEFLHSDLLEDSKYRKIYSFTLKPRTIEDFESMNTYLQTSPSSVFTSKSFCSLQTPDGVHCL
VGFTLTHRRFNYKDNTDLIEFKTLSEEEIEKVLKNIFNISLQRKLVPKHGDRFFTI

SEQ ID NO. 31: *BFY3 cDNA*

ATGCTTTGGAAATTGACGGATAATATCAAGTACGAGGACTGCGAGGACCGTCACGACGGC
ACCAGCAACGGGACGGCACGGTTGCCCCAGCTGGGCACTGTAGGTCAATCTCCCTACACG
AGCGCCCCGCCGCTGTCCCACACCCCCAATGCCGACTTCCAGCCCCCATACTTCCCCCCA
CCCTACCAGCCTATCTACCCCCAGTCGCAAGATCCTTACTCCCACGTCAACGACCCCTAC
AGCCTGAACCCCCTGCACGCCCAGCCGCAGCCGCAGCACCCAGGCTGGCCCGGCCAGAGG
CAGAGCCAGGAGTCTGGGCTCCTGCACACGCACCGGGGGCTGCCTCACCAGCTGTCGGGC
CTGGATCCTCGCAGGGACTACAGGCGGCACGAGGACCTCCTGCACGGCCCACACGCGCTC
AGCTCAGGACTCGGAGACCTCTCGATCCACTCCTTACCTCACGCCATCGAGGAGGTCCCG
CATGTAGAAGACCCGGGTATTAACATCCCAGATCAAACTGTAATTAAGAAAGGCCCCGTG
TCCCTGTCCAAGTCCAACAGCAATGCCGTCTCCGCCATCCCTATTAACAAGGACAACCTC
TTCGGCGGCGTGGTGAACCCCAACGAAGTCTTCTGTTCAGTTCCGGGTCGCCTCTCGCTC
CTCAGCTCCACCTCGAAGTACAAGGTCACGGTGGCGGAAGTGCAGCGGCGGCTCTCACCA
CCCGAGTGTCTCAACGCGTCGCTGCTGGGCGGAGTGCTCCGGAGGGCGAAGTCTAAAAAT

GGAGGAAGATCTTTAAGAGAAAAACTGGACAAAATAGGATTAAATCTGCCTGCAGGGAGA
CGTAAAGCTGCCAACGTTACCCTGCTCACATCACTAGTAGAGGGAGAAGCTGTCCACCTA
GCCAGGGACTTTGGGTACGTGTGCGAAACCGAATTTCCTGCCAAAGCAGTAGCTGAATTT
CTCAACCGACAACATTCCGATCCCAATGAGCAAGTGACAAGAAAAAACATGCTCCTGGCT
ACAAAACAGATATGCAAAGAGTTCACCGACCTGCTGGCTCAGGACCGATCTCCCCTGGGG
AACTCACGGCCCAACCCCATCCTGGAGCCCGGCATCCAGAGCTGCTTGACCCACTTCAAC
CTCATCTCCCACGGCTTCGGCAGCCCCGCGGTGTGTGCCGCGGTCACGGCCCTGCAGAAC
TATCTCACCGAGGCCCTCAAGGCCATGGACAAAATGTACCTCAGCAACAACCCCAACAGC
CACACGGACAACAACGCCAAAAGCAGTGACAAAGAGGAGAAGCACAGAAAGTGA

SEQ ID NO. 32: *BFY3 Amino Acid Sequence*

MLWKLTDNIK YEDCEDRHDG TSNGTARLPQ LGTVGQSPYT SAPPLSHTPN
ADFQPPYFPP PYQPIYPQSQ DPYSHVNDPY SLNPLHAQPQ PQHPGWPGQR
QSQESGLLHT HRGLPHQLSG LDPRRDYRRH EDLLHGPHAL SSGLGDLSIH
SLPHAIEEVP HVEDPGINIP DQTVIKKGPV SLSKSNSNAV SAIPINKDNL
FGGVVNPNEV FCSVPGRLSL LSSTSKYKVT VAEVQRRLSP PECLNASLLG
GVLRRAKSKN GGRSLREKLD KIGLNLPAGR RKAANVTLLT SLVEGEAVHL
ARDFGYVCET EFPAKAVAEF LNRQHSDPNE QVTRKNMLLA TKQICKEFTD
LLAQDRSPLG NSRPNPILEP GIQSCLTHFN LISHGFGSPA VCAAVTALQN
YLTEALKAMD KMYLSNNPNS HTDNNAKSSD KEEKHRK

SEQ ID NO. 33: AS032F (BCZ4 forward primer)
GGAATTCAACATGGACATTGAAGCATATCTTGAAAGAATTG

SEQ ID NO.: 34: AS034R (BCZ4 reverse primer)
GGAATTCCTGGTGAGCTGGATGACAAATAGACAAGATTG

SEQ ID NO.: 35: AS007F (BFY3 forward primer)
GGAATTC*ACC*ATGCTTTGGAAATTGACGGAT

SEQ ID NO.: 36 AS010R (BFY3 reverse primer)
GGAATTCCTCACTTTCTGTGCTTCTCCTCTTTGTCA